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THE RESPONSE OF HYPODYNAMIC MYOCARDIUM TO KNOWN CONCENTRATIONS OF CARDIAC GLYCOSIDES*

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Precise data on the optimal concentrations of cardiotropic or inotropic drugs are few. For this purpose Cattell and Gold (1) have pointed out that the use of isolated heart muscle presents several advantages. Accordingly they have recommended a technique employing the isolated papillary muscle of the right ventricle of the cat's heart. This isolated preparation serves to avoid many complexities which plague the investigator who uses a more complex biological preparation. Furthermore, it permits the testing of extremely small amounts of rare drugs both quantitatively and qualitatively. In addition, the present authors have utilized a method of studying improvement after "systematic tiring" rather than *toxicity* or *equilibration-time* to evaluate the effectiveness of each drug being tested. In this way some assurance is gained that the effect being measured is the desired therapeutic result. Finally, the data can be correlated on a statistical basis so that the results approach the significance of a crude bioassay method.

EXPERIMENTAL PLAN. The experimental approach involved, first of all, the systematic establishment of a hypodynamic state in an isolated papillary muscle of approximately 2 to 10 millimeters length and about one millimeter diameter. This preparation was set up in a chamber similar to that described by Cattell and Gold to record isometric contraction, as illustrated in fig. 1. Similar studies have been made by Krop (2) and by Weeks and Holck (3). Thereupon a systematic procedure was employed in order to induce uniformly the same sort of feeble response in successive preparations. The method of inducing this fatigue as finally adopted included two chief features. First, the muscle was stimulated continuously by a thyatron-controlled electronic device at a constant rate of about 50 times per minute over the course of several hours. During the entire experiment the muscle was allowed a copious supply of pure oxygen which was bubbled constantly through the chamber in which the muscle was housed.

The second feature of the "tiring" process involved the fluid in which the muscle was bathed. This solution was a modified Krebs-Henseleit solution (4), but differed from the original Krebs-Henseleit solution in the following important item: in place of the bicar-

* For the pure substances used in this study the authors are indebted to Dr. Harry Gold, to S. M. Fossel of Sandoz Chemical Works, Inc., and to Dr. K. K. Chen of the Eli Lilly Company.

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† The data in this report were obtained in work in partial fulfillment of the requirements for the doctorate in Pharmacology at Yale University.

bonate buffer an 0.154 M phosphate buffer was substituted, to bring the final solution to pH 7.2. In this way bicarbonate ions were completely excluded except for those accumulating as a result of metabolic processes. Finally the test solutions were introduced *seriatim* into the chamber after reinforcement with known concentrations of standard or unknown inotropic drugs. The range of concentrations studied in the case of crystalline drugs was approximately 1.0 to 40.0 micrograms per cent. These solutions ordinarily were so arranged that at successive stages of the experiment increasing concentrations of the drugs were employed in cascade or staircase fashion. By judicious interchange of standard and unknown drugs at successive stages in the series of increasing potency, it was possible to compare the inotropic effect of standard and unknown solutions on the same bit of myocardium.

EXPERIMENTAL PROCEDURE. After the muscle tension had been adjusted to maximal excursion it was left under constant stimulation for a matter of one to six hours until a severe decline in contractility had occurred, e.g., to one centimeter's excursion as against

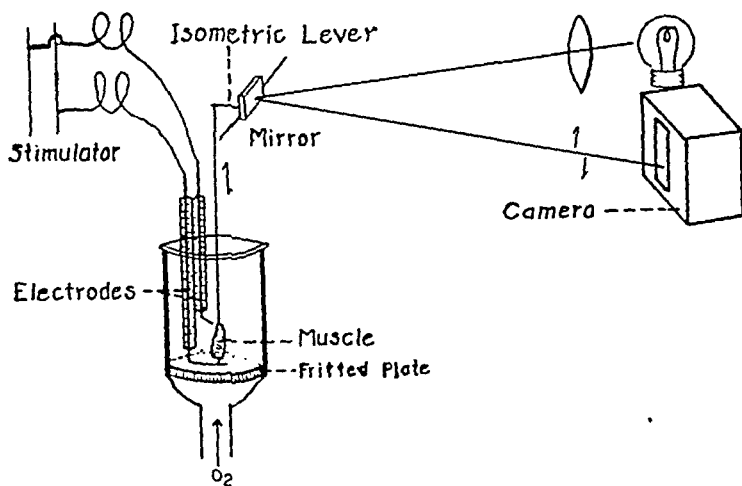


FIG. 1. SCHEMATIC DIAGRAM OF THE MUSCLE CHAMBER, SHOWING THE RELATION OF THE FRITTED GLASS PLATE TO THE MUSCLE AND ELECTRODES

five centimeter's original amplitude in the camera record. Then the solution in the chamber was replaced with a fresh solution containing a known concentration of the drug under test. Thereafter, a short record of the excursion was taken at intervals of about ten minutes each. In the case of ouabain it was found that after an hour's equilibration with such a solution the muscle had attained a constant increased amplitude, provided that not too large an increase in the concentration of drug had been imposed. Thereupon the solution was removed and another solution containing a higher concentration of the drug was substituted immediately.

Successively increasing concentrations of drug were utilized in this manner until eventually the muscle excursion declined and the muscle ceased to contract in the "toxic" range of concentration. It was found convenient to use logarithmic multiples of 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 in adjusting the concentration ranges. In other words, the successive concentrations of ouabain ordinarily used, expressed in micrograms per cent, were respectively, 1.0, 1.6, 2.5, 4.0, 6.3, 10.0, 15.8, and 25.0.

Representative protocol. In fig. 2 is illustrated a representative protocol performed on muscle No. 9. This preparation was only 2 mm. long (when free) and had a dry weight of 0.6 mgm. It was stimulated at the rather slow rate of 38 times per minute. The figure shows the history of the preparation for about eleven hours. Up to a concentration of 15.8 micrograms per cent there was a steady increase in the amplitude of muscular contraction: so that the muscle was practically restored to its original status. Above 15.8 micrograms per cent, however, the muscle became poisoned by the drug and the contractions fell off rapidly at a concentration of 25.0 micrograms per cent.

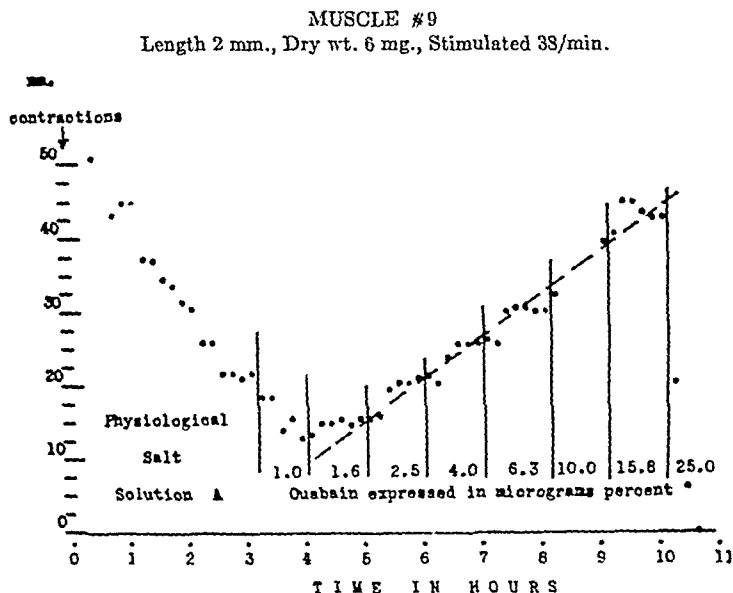


FIG. 2. THE "FATIGUING" PROCESS IN PHOSPHATE BUFFER CAN BE REVERSED IN ROUGH MIRROR IMAGE BY A SUITABLE CASCADE OR STAIRCASE SERIES OF INCREASING LOGARITHMIC CONCENTRATIONS OF POSITIVE INOTROPIC DRUG

Phosphate vs. Bicarbonate Buffer. As illustrated in fig. 3, in bicarbonate buffer the cat's papillary muscle may continue to respond without obvious change in contractility for more than a day. Under such circumstances, the muscle may fail to respond well to inotropic drugs in the therapeutic range of concentration. It is possible by various means to make the muscle hypodynamic without permanently injuring it. It will be seen from fig. 4 that one such method is to replace the bicarbonate buffer (B) by the phosphate buffer (A). This modification does not permanently alter the muscle since it quickly recovers when the phosphate buffer is replaced by a bicarbonate buffer. In the case of the hypodynamic muscle #4E, contractility was partially restored while in the phosphate buffer by the use of a cardiotonic glycoside.

MUSCLE #3A

Length 4 mm., Dry wt. .8 mg., Stimulated 68/min.

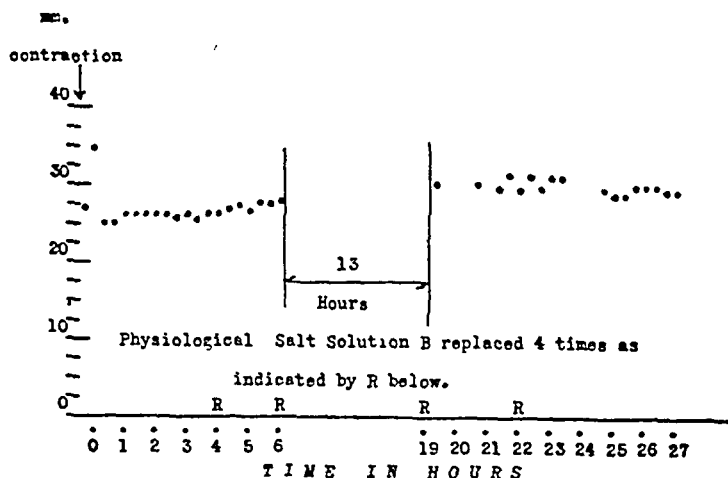


FIG. 3. WHEN A PAPILLARY MUSCLE IS PRESERVED IN BICARBONATE BUFFER AT pH 7.4, IT IS FREQUENTLY DIFFICULT TO INDUCE A HYPODYNAMIC STATE
The gas mixture for equilibration contained 95% oxygen and 5% carbon dioxide.

EFFECT OF CHANGE OF MEDIUM

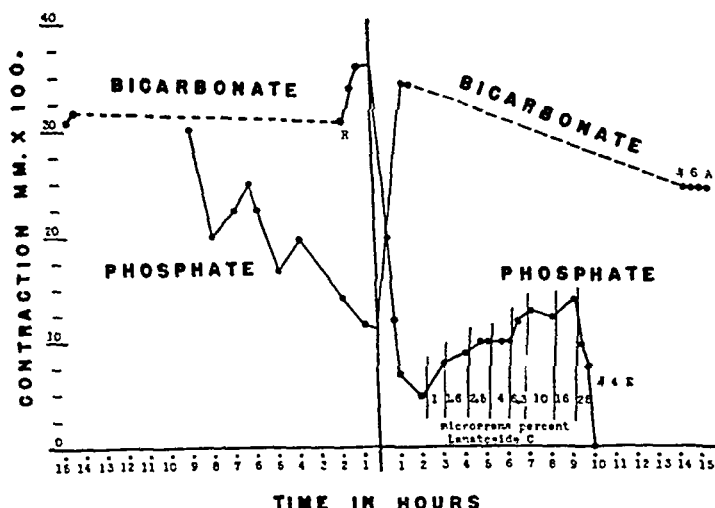


FIG. 4. CONTRACTIONS ARE CONSISTENTLY HIGHER IN BICARBONATE BUFFERED SOLUTIONS THAN IN PHOSPHATE BUFFERED SOLUTIONS

The ionic make-up and pH of the two solutions are otherwise similar.

EXPERIMENTAL DATA. When the photographic paper was developed, ordinarily it showed five records at 100-fold magnification covering each hourly period. Usually each of the isolated records contained about ten contractions, which were rather uniform. The maximal level of contraction reached in each of the five periods was measured from the base line. Four typical series of responses attained with papillary muscles at hourly intervals are given in table 1, together

TABLE 1
Comparative potencies of four inotropic drugs

	OUABAIN*			DIGITOXIN			COUMINGINE†			K-STROPHANTHOSIDE		
	Time in hours	Conc. $\mu\text{g}\%$	Re-sponse mm. $\times 100$	Time in hours	Conc. $\mu\text{g}\%$	Re-sponse mm. $\times 100$	Time in hours	Conc. $\mu\text{g}\%$	Re-sponse mm. $\times 100$	Time in hours	Conc. $\mu\text{g}\%$	Re-sponse mm. $\times 100$
Fatigue	-6	0			0	60						
	-5	0			0	39	0	81		0		24.0
	-4	0			0	35	0			0		31.5
	-3	0	61		0	29	0	54		0		34.8
	-2	0	30		0	26	0	50		0		35.8
	-1	0	15		0		0	39		0		32.0
	0	0	10		0	32	0	31		0		37.5
Positive inotropic response	1	1.0	13	1	1.0	32	0.5	1.0	27	1	1.0	41.5
	2	1.6	17	3	1.6	32	1.0	1.6	26	3	1.6	38.0
	3	2.5	29	4.5	2.5	28	1.5	2.5	29	4	2.5	37.4
	4	4.0	43	5.0	4.0	30	2.5	4.0	49	5	4.0	47.0
	5	6.3	63	(6.3 for 8 hours)			3.5	6.3	68	7	6.3	66.2
	6	10.0	81	15.0	6.3	24				8	10.0	76.8
Toxic effect	7	15.8	43	16.5	10.0	40	4.5	10.0	52	9	15.8	80.0
	7.5	25.0	0	18.0	15.8	69	5.5	15.8	0	9.5	25.0	27.0
				19.0	25.0	78						
				19.5	31.6	3						

* Ouabain Reference Standard (about 11% water).

† Coumingine Hydrochloride (about 7% HCl).

with the concentrations of the four drugs involved, i.e., ouabain, digitoxin, coumingine, and k-strophanthoside.

In table 2 are recorded the responses of 9 muscles to ouabain, with reference to the threshold, maximal and toxic concentrations involved. It will be observed that the slopes of the individual semi-log response curves vary considerably in these several preparations. This variation may be partly due to differences in the shape, size and nutritional state of the individual muscles.

STATISTICAL APPLICATION. For the crude assay of small amounts of rare drugs this method recommends itself because successive responses can be observed in the same slip

of myocardial tissue. As a first crude approximation, the respective responses to *known* concentrations can be plotted against the logarithms of the concentrations, and the successive points connected by straight lines. Then by simple interpolation each response to an *unknown* concentration can be converted into a concentration-value by direct reading from the crude graph. For example, the odd-numbered data in table 2 were plotted for muscles numbered 9, 10, and 12. The alternate (or even-numbered) responses were considered as "unknown" and were converted into experimentally-determined values by simple interpolation. Actually the designated "unknown" concentrations in micrograms per cent were respectively 1.6, 4.0, and 10.0. For muscle No. 9 the interpolated values were 1.6, 3.7, and 9.6. For muscle No. 10 the interpolated values were 1.4, 3.7, and 10.6. For muscle No. 12 the interpolated values were 1.8, 4.4, and 10.3. The averaged values were 1.6, 3.9, and 10.2. These, in percentage of known, become 100.0, 97.5, and 102.0. The grand average is 99.8 per cent.

TABLE 2

Summary of data on the response of the papillary muscle to ouabain

MUSCLE	PRELIMINARY PERIOD	RESPONSE TO OUABAIN			SLOPES	LENGTH	DRY WEIGHT
		Threshold	Maximal	Toxic			
						millimeter	milligram
#9	2½ hrs.	1.6 µg%	15.8 µg%	25 µg%	27.0	2	0.6
#12	1½ hrs.	1.0	10.0	15.8	27.6	5	1.29
#10	3 hrs.	1.0	10.0	15.8		7	1.49
#11	5½ hrs.	1.0	6.3	10.0	5.12	7	0.77
#13	4½ hrs.	1.0		15.8		7	3.0
#14	3 hrs.	1.0		15.8		9	2.58
#16	3½ hrs.				19.0	6	2.69
#1A	6 hrs.			12.0	23.4	5	1.1
#4A	21 hrs.	1.6	4.0*		13.75	5	1.47
Average.....			10.7 µg%	15.7 µg%			

* Excluded because muscle failed to respond to higher doses.

It is of some interest, however, to be able to reduce to a common denominator independent assays performed on different muscles so that the results of several experiments can be combined and appropriately weighted statistically. To this end the procedure tentatively adopted has been to chart the data in terms of the maximal response which is set at 100%. For each concentration tested the percentile response is plotted against the log-concentration of the inotropic substance as illustrated in fig. 5. It will be noted that through this device the various muscles tend to group together. Moreover, the concentration-response relationship is essentially linear when plotted as a semi-log function. In a typical instance, for example, when an individual experiment was analyzed for the effect of linear regression and the effect of curvature, respectively, the analysis of variance gave an F value of 1581 for linearity as against 9.6 for curvature.

In a typical experiment, arranged according to Bliss (5), two stock solutions of ouabain were compared. The first, designated as the standard, contained 6.67 milligrams per liter; the second, designated as the unknown, contained 10.0 milligrams per liter. In the actual procedure the dual samples used were 0.1 and 0.2 ml., respectively, of each stock solution. These four amounts were

added successively to the 25 ml. of Krebs-Henseleit solution in the chamber. The comparative potency was calculated as a single two-dose assay. The "unknown" potency (in per cent of standard) was estimated to be $143\% \pm 10.4$, whereas 150.0% was expected. It is interesting that crude interpolation of the raw data graphically gave the result 146.2% .

Pooled data. When the data shown by the solid lines in fig. 5 are combined and analyzed statistically, the following values were obtained for the even-numbered concentrations designated as "unknown" as previously discussed. For muscle No. 9, the potency was $97.9 \pm 10.6\%$; for muscle No. 10, $95.5 \pm$

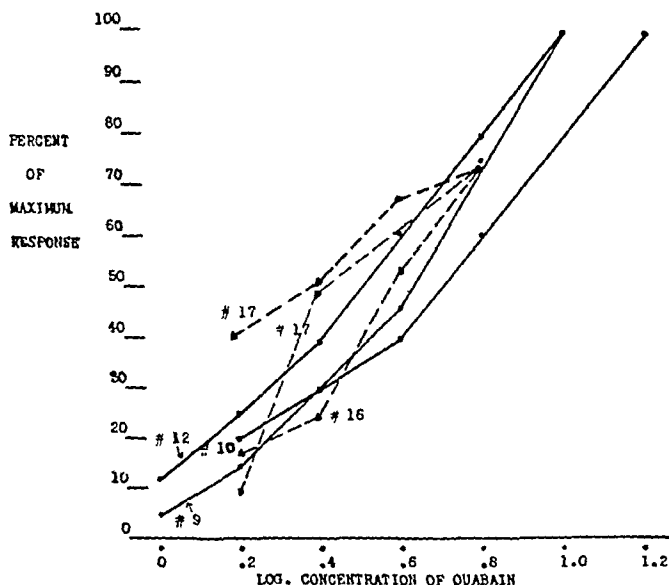


FIG. 5. EFFECT OF OUABAIN UPON SIX INDIVIDUAL PAPILLARY MUSCLES

The solid lines are plotted as per cent of the maximal response. The broken lines are plotted on the assumption that 75% of the maximal response is produced by a concentration of 6.3 micrograms per cent.

18.5% ; for muscle No. 12, $110.3 \pm 10.7\%$; and for the pooled data, a weighted mean potency of $103.4 \pm 5.9\%$. The authors are indebted to Miss Barbara Bartels for these calculations. The statistical method used was adapted from Bliss and H. P. Marks (6) and Miller, Bliss and Braun (7).

Isodynamic Substitution of Glycosides. From the response of two separate muscles, it was determined that digitoxin must be present in a concentration approximately 2.5 times that of ouabain to produce an equivalent inotropic effect. This potency factor of 2.5 could then be verified more precisely on a single muscle by substituting for alternate concentrations of ouabain an isodynamic concentration of digitoxin. For example, with a certain muscle four

responses were plotted against concentrations of ouabain ranging from 1.2 to 10.0 micrograms per cent. By interpolation, the respective responses of four intermediate concentrations of digitoxin were transposed into equivalent concentrations of ouabain. The results were 2.5 times 128%, 98%, 93% and 83%. The average value was 2.5 times 101%. A more precise evaluation was obtained by calculating statistically the response as a linear regression, according to the equation:

$$\log \text{percentile-response} = K \log (\text{concentration} + C).$$

By this method the potency of digitoxin in terms of ouabain was found to be 39.8% \pm 4.1 (2.51 digitoxin \approx 1.0 Ouabain Reference Standard).

This method is based upon certain fundamental observations indicating that isodynamic solutions of different glycosides may be interchanged rapidly without summation of possible residual effects. For example, a mixture of 4 micrograms per cent of ouabain with 10 micrograms per cent of digitoxin produces the same contraction as 8 micrograms per cent of ouabain alone. Similarly, when 4 micrograms per cent of ouabain is rapidly substituted for 10 micrograms per cent of digitoxin, no change in amplitude results; whereas 8 micrograms per cent of ouabain subsequently causes a marked increase in amplitude. The time required for equilibration ceases to be a difficult complication of such experiments because only minor changes in contraction are induced by the judicious interchange of nearly isodynamic solutions.

DISCUSSION. In this report the increased amplitude of myocardial contraction has been employed to evaluate the potency of inotropic drugs in terms of concentration. As pointed out by Krop (2), the use of toxicity for this purpose may be misleading, as in the case of barium ions. Precise comparisons of various cardiotropic substances will be published elsewhere (8). If for the sake of illustration, however, the single experiments presented in Table II are accepted at their face values, the following potencies are found. On the basis of the weight required to produce a response 80% of maximal, if ouabain = 1.00, digitoxin = 2.55, coumagine hydrochloride = 0.78, and K-strophanthoside = 1.27. On a molecular basis, if ouabain = 1.00, these values become for digitoxin 2.2, coumagine 0.96, and K-strophanthoside 0.89. For this calculation the following values for the respective molecular weights were employed: for ouabain (anhydrous) 648.7; for digitoxin 764.5; for coumagine hydrochloride 528.0; and for K-strophanthoside 926.5. It is interesting that the difference between ouabain and digitoxin resembles that reported by Gold (9) for human assays. It will also be noted that according to the present experiments the optimal concentration for ouabain is about two-thirds of the toxic concentration. A similar relationship was obtained by Krueger and Unna (10) who utilized the doses in whole cats required to produce cardiac irregularities and death, respectively.

As regards the substitution of phosphate for bicarbonate buffer, Warren (11) has pointed out that the rate of energy consumption of various mammalian tissues is higher in bicarbonate—than in phosphate—Ringer's medium. Indeed

Buchanan and Hastings (12) have stated that carbon dioxide may no longer be considered as simply an end product of metabolism, but rather as an essential component of certain metabolic reactions. The withdrawal of this essential substance from the medium, therefore, might be expected to favor the production of a hypodynamic state. As might be predicted, fresh muscles (not yet hypodynamic) fail to respond to non-toxic concentrations of inotropic drugs.

SUMMARY AND CONCLUSIONS

1. The relative potencies of ouabain, digitoxin, coumestrol and K-strophanthoside have been determined approximately by their inotropic effects on the isolated papillary muscle of the cat's heart. An experimental procedure is described for verifying isodynamic concentrations of two such drugs on the same muscle, with appropriate statistical analysis. The method suggested involves prolonged electrical stimulation in the absence of bicarbonate ions.

2. The semi-logarithmic concentration-response curve can be used to assay unknown drugs by distributing standard and unknown concentrations in cascade or staircase fashion along the dosage-response curve. Several isolated preparations may be correlated by expressing the results in terms of percentile response of the maximum contraction observed in each case.

The authors wish to acknowledge helpful advice from Drs. McKen Cattell, Harry Gold and Stephen Krop.

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COMPARISON OF PRESSOR ACTION OF ALIPHATIC AMINES

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The resemblance of aliphatic to aromatic amines in raising blood pressure has been repeatedly observed and reviewed (1-4). Recently, there has been a renewal of interest in those aliphatic amines which have not been studied before (5-7). Indeed, certain compounds are suitable for clinical use (8-9). Pharmacological studies on other newer products have also been reported (10-11). The present investigation concerns a comparison of the pressor activity of thirty-nine aliphatic amines, prepared by Dr. E. Rohrmann and Mr. H. A. Shonle of our own laboratories.

PROCEDURE AND RESULTS. As before (12-13), experiments were carried out in pithed dogs. The animals, 176 in number, weighed from 5.1 to 11.8 kg., averaging 7.5 kg. It was soon disclosed that the majority of the aliphatic amines caused tachyphylaxis after the first intravenous injection. It was, therefore, necessary to evaluate the pressor action of various substances in individual dogs with epinephrine as a standard. Comparisons were made only with reference to the intensity of action; namely, the peak of rise of each amine was matched with that of a suitable dose of epinephrine chloride. Groups of 3 to 8 dogs were used for each compound. All the amines, in form of the sulfate, were water soluble and in 2 to 4 per cent solution. Injections were always made by the femoral vein.

The results are presented in table 1, together with the chemical names of the thirty-nine amines studied. The average epinephrine equivalents are shown in the last column. The analysis of the results with reference to the chemical structure can be made as follows:

(1) NH_2 on C_1 of *n*-alkanes. If consideration is given to those normal hydrocarbons in which the amino group is attached to the terminal C-atom, it becomes at once apparent that 1-amino-hexane is more powerful than 1-amino-pentane, 1-amino-heptane, and 1-amino-octane.

(2) *Change of position of NH_2 .* When NH_2 is attached to the second C-atom of *n*-alkanes, it invariably becomes a stronger product than when NH_2 is attached to the terminal C-atom. Thus, 2-amino-pentane is almost twice as active as 1-amino-pentane. Similarly, 2-amino-heptane is fully eleven times as active as 1-amino-heptane. Both 2-amino-hexane and 2-amino-octane are definitely more active than 1-amino-hexane and -octane, respectively. If the amino group is on C_3 or C_4 , the activity of these compounds is less than that of either 1-amino or very much less than that of 2-amino compounds, as shown in the heptane series. These data indicate that 2-amino substitution will result in relatively more potent substances.

(3) *Methylation of *n*-alkanes when NH_2 is on C_1 .* In the pentane series, the substitution of a methyl group at C_4 is better than at C_3 , since 1-amino-4-methyl-pentane is much more powerful than 1-amino-pentane. When two methyl

TABLE 1

Comparison of pressor activity of aliphatic amines in piloth dogs

COMPOUND			NUMBER OF DOGS USED	RANGE OF DOSES TESTED	AVERAGE EPINEPHRINE EQUIVALENT FOR 1 MG. OF AMINE*
NUMBER	Side chain	Stem nucleus			
1	1-Amino-3-methyl-	butane	4	20-100	0.283
2	1-Amino-	pentane	5	20-60	0.210
3	1-Amino-3-methyl-		5	20-40	0.193
4	1-Amino-4-methyl-		5	25-50	0.530
5	1-Amino-3,4-dimethyl-		3	12-40	1.720
6	2-Amino-		5	40-60	0.335
7	2-Amino-4-methyl-		5	15-20	0.840
8	2-Amino-4,4-dimethyl-		3	50-60	0.093
9	1-Amino-	hexane	5	12-20	0.766
10	1-Amino-3-methyl-		2	20	0.103
11	1-Amino-3,5-dimethyl-		3	20-50	0.660
12	2-Amino-		5	12-20	0.901
13	2-Amino-3-methyl-		5	20-30	0.858
14	2-Amino-4-methyl-		6	6-10	3.520
15	2-Amino-5-methyl-		5	10-15	1.560
16	2-Amino-	hexane-5	5	20-54	0.193
17	2-Amino-5-methyl-		5	28-60	0.504
18	1-Amino-	heptane	8	7.5-30	0.276
19	1-Amino-3-methyl-		5	20-30	1.220
20	2-Amino-		5	5-12	3.100
21	2-Amino-3-methyl-		5	10-40	0.520
22	2-Amino-4-methyl-		5	20-30	0.786
23	2-Amino-5-methyl-		6	20-60	0.960
24	2-Amino-6-methyl-		6	10	2.620
25	2-Amino-3,6-dimethyl-		5	20-30	0.910
26	2-Amino-4,6-dimethyl-		3	40-50	0.193
27	2-Amino-5-ethyl-		3	20-60	0.358
28	2-Amino-4,6,6-trimethyl-		3	40-50	nil
29	3-Amino-		7	20	0.149
30	3-Amino-6-methyl-		3	20-60	nil
31	4-Amino-		5	10-20	0.045
32	4-Amino-2,6-dimethyl-		3	20-40	nil
33	2-Amino-6-methyl-	heptene-5	5	50-100	0.391
34	1-Amino-	octane	4	40-100	nil
35	2-Amino-		4	15-40	0.510
36	2-Amino-4-methyl-		3	50-50	0.475
37	2-Amino-3-hydroxy-		5	40-50	0.122
38	3-Amino-		3	40-50	0.142
39	2-Amino-4-methyl-	decane	3	50	0.242

* In form of the sulfate.

groups are introduced to C_3 and C_4 in the molecule of 1-amino-pentane, the resulting compound is very much more potent. However, if the methylation takes place simultaneously at C_3 and C_5 in the molecule of 1-amino-hexane, the resulting compound is weaker than 1-amino-hexane. Methylation at C_3 alone is followed by a substantial decrease of pressor activity. On the other hand, methylation on the same C-atom in the molecule of 1-amino-heptane results in an increase of potency.

(4) *Methylation, ethylation, or hydroxylation of n-alkanes when NH_2 is on C_2 .* In the 2-amino-pentane series, methylation on C_4 gives rise to a very much more active compound. If, however, a second methyl group is introduced into C_4 , there is a great decrease in activity. In the 2-amino-hexane series, methylation at C_4 is very much better than methylation at C_3 , since 2-amino-4-methyl-hexane is the best of the entire group of thirty-nine amines. Methylation at C_5 results in a compound more active than that with a CH_3 at C_3 , but not as potent as that with a CH_3 at C_4 . In the 2-amino-heptane series, methylation at C_5 confers more activity on the compound than at any other position, although it is weaker than the parent substance; namely, 2-amino-heptane. Introduction of two methyl groups at C_3 and C_6 , and C_4 and C_6 , greatly weakens the pressor activity of 2-amino-heptane. Ethylation at C_5 has a greater unfavorable influence on the blood pressure than methylation at the same position. When three methyl groups are introduced, one at C_4 and two at C_6 , the resulting compound is completely devoid of action. Of the 2-amino-octane series, neither methylation at C_4 nor hydroxylation at C_3 has a favorable influence on the pressor action exhibited by 2-amino-octane itself.

(5) *Methylation of n-heptane when NH_2 is on C_3 or C_4 .* Introduction of a methyl group at C_6 makes 3-amino-heptane entirely inactive. Similarly, introduction of two methyl groups at C_2 and C_6 makes 4-amino-heptane completely lose its effect on blood pressure.

(6) *Unsaturation.* There are three unsaturated amines in the series. It is clear that the presence of a double bond decreases the activity, since 2-amino-hexane is more powerful than 2-amino-hexene; 2-amino-5-methyl-hexane, more active than 2-amino-5-methyl-hexene; and 2-amino-6-methyl-heptane, more active than 2-amino-6-methyl-heptene.

(7) *Total number of C-atoms.* The most active member of the entire series is 2-amino-4-methyl-hexane. The next in order is 2-amino-heptane. Both amines have a total number of seven C-atoms. This is probably the optimal length of the chain showing the highest pressor activity. Any smaller number of C-atoms, as in case of 1-amino-3-methyl-butane, or any increase of C-atoms, as in case of 2-amino-4-methyl-decane, results in lowering of pressor activity.

The duration of action was not systematically compiled as the intensity of action. In general, however, it runs parallel with the latter; that is, whenever a compound has an intense action on blood pressure, it is also long acting.

The series under investigation does not represent a complete variation of each stem nucleus, so that it is impossible to interpret the full significance of each change in position of either the NH_2 group or the methyl group. The results

recorded in table 1 show some variance with those published by Rohrmann and Shonle (6). For example, the order of activity of 2-amino-4-methyl-heptane, 2-amino-5-methyl-heptane, and 2-amino-6-methyl-heptane is completely reversed in the aforementioned article. It may be stated, however, that the results in the Rohrmann-Shonle paper (6) were preliminary in nature, obtained in one or two cats, and limited to only one arbitrary dose for each compound. The data in this presentation should be considered more reliable, since more animals were used and various dose ranges employed, aside from the fact that dogs instead of cats were used.

SUMMARY

The pressor activity of thirty-nine aliphatic amines has been compared in pithed dogs. The most active compounds are 2-amino-4-methyl-hexane and 2-amino-heptane. Presentation of results has been made in relation to chemical structure. The amino groups on C_2 appears to have the most favorable influence on the pressor activity.

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DIGITALIS III

THE RELATIONSHIP BETWEEN THE POTENCY AND BALJET REACTION OF THE GLYCOSIDES OF DIGITALIS¹

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In recent studies we have been interested in the application of the Baljet color reaction to the assay of digitalis preparations (1) and the assay of tablets of digitoxin (2). A consideration of the problems arising in these studies led us to the conclusion that a more extensive examination of the digitalis glycosides with respect to this color reaction would prove of value especially since the methods of preparation and isolation of pure glycosidal substances have undergone a marked advance in recent years.

In this communication we report the results obtained in the examination of the principal leaf-glycosides of *digitalis purpurea* and *digitalis lanata*. These glycosides are digitoxin, gitoxin and gitalin (amorphous), lanatosides A, B, and C, and digoxin.²

The experimental method for examining these substances was essentially that employed in establishing our method for the assay of tablets of digitoxin (3). Stock solutions of the seven glycosides in absolute methanol were prepared and by proper dilution with the same solvent, solutions containing 5, 10, 15, 20, 25 and 30 mg. respectively per 100 cc. were obtained for each glycoside with the exception of gitoxin which showed a maximal solubility of approximately 20 mg. per 100 cc.

The development of the Baljet color reaction with respect to time was determined for each of these 40 solutions using absorption cells having a light path of approximately 25 mm. and a photoelectric colorimeter in the optical region of 525 millimicrons. Measurements of the optical density were made at 10 minute intervals over a period of 2 hours. These data were converted to graphical form by plotting the optical density against the time. Each of these curves followed a very similar pattern. The optical density reached a maximum value in each case approximately 40 minutes after the reagent had been added. Throughout the remainder of the 2 hour period the optical density either remained approximately constant or showed a very gradual decrease. Checks on these curves were readily obtained within the accuracy of the measuring instrument and normal variations in room temperature appeared to have no significant effect.

In figure 1 a summary of these results is shown graphically by a curve for each of the *purpurea* glycosides in which the 40 minute optical density reading is plotted against the concentration in mg. per 100 cc. Similarly in figure 2 the

¹ The expense of this investigation was defrayed in part by a grant from the Board of Trustees of the United States Pharmacopoeial Convention.

² Specimens of these glycosides were obtained from the following sources: the three lanatosides from Sandoz Chemical Works, Inc., digoxin from the Wellcome Research Laboratories, digitoxin from the Ladox Laboratories, gitoxin from Hoffmann-La Roche and Company and gitalin from Rare Chemicals, Inc.

corresponding curves for the three lanatosides are shown. The curve for digoxin is included in each figure.

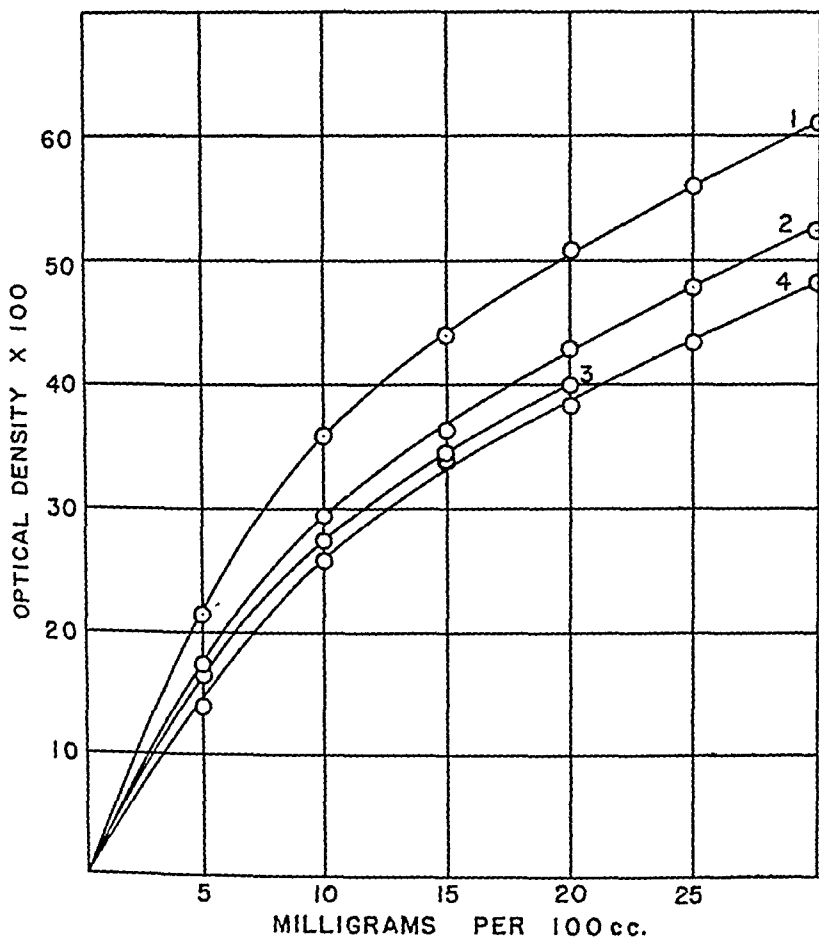


FIG. 1

- 1—digitoxin
- 2—digoxin
- 3—gitoxin
- 4—gitalin

The curves in figure 1 show that the color enveloped by digitoxin is considerably more intense than that developed by either gitoxin or gitalin, the latter two exhibit approximately the same color intensity. The ratios of the color intensities among these three substances are in the same order and the same general

magnitude as the bio-assay values cited in the literature. In other words, the optical density is a direct function of the cardiotonic value of the three *purpurea* glycosides thus substantiating the observations of Baljet (4) and, later, of Knudsen and Dresbach (5). It seems highly probable that for mixtures of the three glycosides the same relationship would hold. While it is realized that

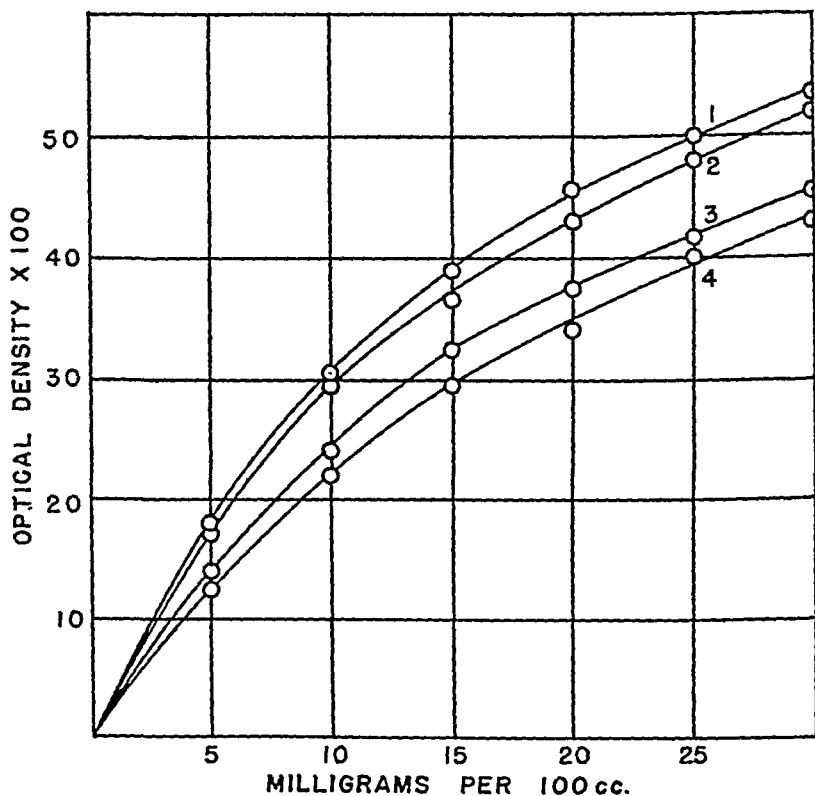


FIG. 2

- 1—lanatoside A
- 2—digoxin
- 3—lanatoside B
- 4—lanatoside C

caution must be exercised in interpreting these observations, which are carried out under relatively simple conditions, in terms of the procedure which we have developed for the assay of *digitalis purpurea* preparations, it is believed that the data offer strong support to the validity of that procedure.

Baljet (6) suggested that his reaction depended upon the presence of the lac-

tone group in the glycoside molecule and more recent work, particularly by Jacobs (7) and his co-workers, has shown that the presence of this group is essential to cardiotonic activity.

An interesting question immediately arises. Why do these three glycosides each containing the unsaturated lactone group, having similar molecular weights and a close similarity in general structure, show a marked difference in color intensity with the Baljet reaction? From his photometric studies of the Baljet reaction with glycosides and genins containing the unsaturated lactone groups Hagemer (8) has concluded that equi-molar solutions of these substances after treatment with alkaline sodium picrate solution will have identical coefficients of extinction provided the substances are pure. While the question of purity is undoubtedly of great importance we have approached the problem from a different point of view.

TABLE 1

	C-12	C-14	C-16
Digitoxin.....		OH	
Gitoxin.....		OH	OH
Gitalin.....		OH	OH
Digoxin.....	OH	OH	

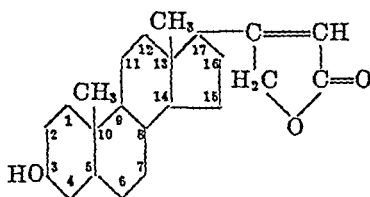


Fig. 3

In table 1 are listed the positions of the hydroxyl groups of the three *purpurea* glycosides and digoxin.

These positions can be readily located by reference to the basic ring structure of the genins shown in figure 3.

In a private communication, Dr. Walter Jacobs of the Rockefeller Institute has suggested to us that the fact that the hydroxyl at C-16 is considerably more reactive than that at C-14 might explain the difference in response of these glycosides toward the Baljet reaction. Pursuing this thought, we have developed an explanation which appears to be in accord with observed facts.

It is known that the aglycones of the digitalis glycosides when treated with alcoholic alkali undergo a characteristic rearrangement leading to the formation of saturated isogenins and these would not be expected to react with the alkaline picrate. In figure 4 this rearrangement for digitoxin in the molecule fragment is shown.

It will be noticed that the isogenin contains a six-membered ring resulting from a rearrangement of the hydrogen atom of the hydroxyl group at C-14.

In gitoxin and gitalin the location of the hydroxyl groups is the same, namely at C-14 and C-16 and therefore each of these substances could form an isogenin similar to that of digitoxin as just described. In addition, however, there is the possibility of another isogenin being formed as shown in figure 5.

The oxygen of the hydroxyl group at C-16 has become a part of a five-membered ring which may be regarded as more stable than the six-membered ring shown in figure 4.

Although digoxin is a degradation product of lanatoside C it is closely related in chemical structure to the three *purpurea* glycosides. It has a hydroxyl group at C-14 as in the case of digitoxin and could be expected to form an isogenin

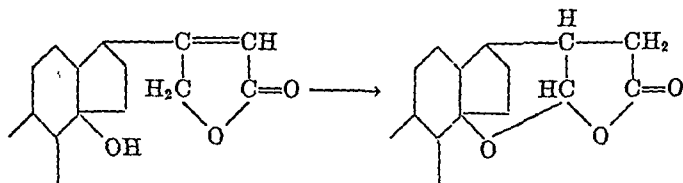


FIG. 4

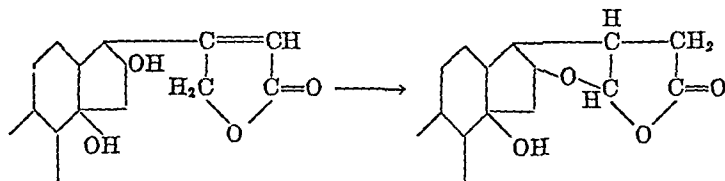


FIG. 5

similar to that shown in figure 4. However, there is also the possibility of the formation of a second isogenin as shown in figure 6.

Again a six-membered ring is formed which includes the oxygen of the hydroxyl group at C-12.

From the foregoing discussion it is clear that if we assume that the tendency of the glycosides to form isomers is similar to that of the genins and that these isomerizations do take place under the conditions required by the Baljet test we have an explanation for the differences observed in that test. For each molecule of isomer formed there is one less molecule of the lactone structure available for reaction with the Baljet reagent. The spatial arrangement of the various ring structures in the glycoside molecules concerning which our information is still very limited, might be expected to be an important factor also. These observations are so striking that one is tempted to postulate that in the administration of the three *purpurea* glycosides isomerization processes take place in the biological test object and thus account for differences in activity. However,

the behavior of digoxin, which is among the most potent of the digitalis glycosides, contradicts this view.

Referring to figure 2 in which the curves for the three lanatosides and digoxin are given the same general pattern is observed as in the case of the *purpurea* glycosides. There is a close parallelism. Lanatoside A, which contains the same genin as digitoxin, shows a markedly higher Baljet test than lanatoside B, which contains the same genin as gitoxin, and lanatoside C. The latter two are of the same order of magnitude while digoxin, a degradation product of lanatoside C, falls somewhat below the value of lanatoside A. If an attempt is made to correlate these data with the corresponding cardiotoxic values it is readily seen that the Baljet test is not applicable to the assay of *lanata* preparations or for simple mixtures of the pure *lanata* glycosides.

Thus far all of our considerations have been based on solutions of the glycosides made up on a weight-volume basis since it is the system with which the bio-

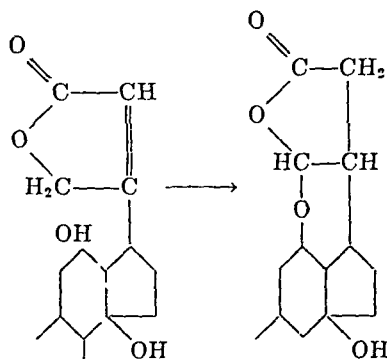


FIG 6

assayist is particularly concerned. However, since the Baljet test apparently involves the direct chemical reaction of saturation of the lactone group of the glycoside with simultaneous reduction of the pieric acid to pieramic acid or sodium pieramate, the chemical aspects of the test can be more readily examined if the glycoside concentrations are expressed in molarities.

In figure 7 we have taken the data of figures 1 and 2 and plotted the optical density against the concentration in micro-moles per 100 cc. Perhaps the most striking feature of these curves is the superposition of the curves for lanatoside A and digitoxin. As has been noted before each of these substances contains the same aglycone and it is therefore obvious that the differences between these two glycosides in the sugar portion of their molecules has no effect on the Baljet test. The points corresponding to digoxin and lanatoside B fall upon the same curve and similarly, those for lanatoside C and gitoxin fall upon the same curve. These relations are exactly the reverse of what might be expected. The curve for digoxin, which is a degradation product of lanatoside C and contains the same

genin, should coincide with the curve for lanatoside C. On the same basis the curves for gitoxin and lanatoside B should coincide. We have checked these values several times in order to eliminate any obvious errors in procedure or technique. No explanation for these observations is offered at this time. The curve for gitalin takes an unique position in that this glycoside shows definitely the lowest degree of activity toward the Baljet reagent. No attempt is made to interpret this curve since the specimen used in this study is described by its manufacturer as a mixture of gitalin and gitoxin. The molecular weight of gitoxin is 781 and that of gitalin is 669. We have used the latter value in com-

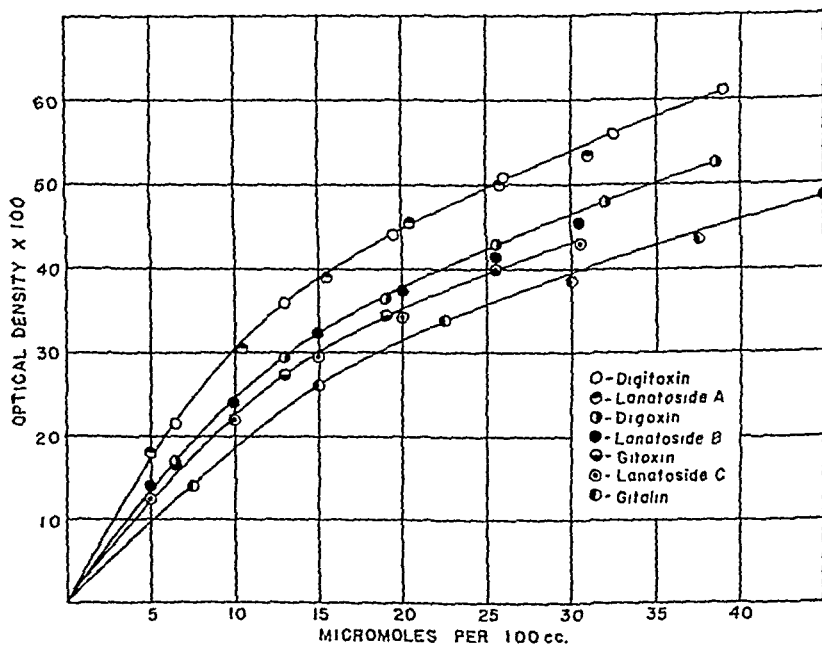


FIG. 7

puting the values in figure 7. Further information concerning the relative amounts of these glycosides present in the mixture would be very helpful. It is worthy of note, however, that the curves for this substance and for gitoxin very closely coincide when the concentrations are expressed on a weight-volume basis. (figure 1.)

In all of the curves shown in figures 1, 2, and 7, it is apparent that there is a marked divergence from Beer's law in the behavior of the solutions. This divergence can also be satisfactorily explained on the basis of isomerization.

We may assume that the intensity of the Baljet reaction is proportional to the amount of glycoside (lactone) present. On the other hand we may assume that

the degree of isomerization is not directly proportional to the concentration of the glycoside. If the observed intensity of the Baljet reaction is regarded as the resultant of these two factors, and since one of them does not show direct proportionality to the concentration, the resultant likewise will not.

We have previously suggested that caution must be used in attempting to interpret the results obtained from these relatively simple procedures in terms of the much more complex conditions which prevail in our method for the assay of digitalis preparations. It is believed that the data presented in this paper are of much assistance in evaluating the validity of that procedure. When one considers the high degree of chemical complexity of the solutions which the method attempts to assay we are not at all surprised that the method fails in some cases. On the other hand it is a source of amazement to us that the method is valid in most cases.

Additional evidence of the complexity of digitalis preparations and the pure principles is apparent in other recent publications. From the examination of digitoxin, digitoxigenin, gitoxin and lanatoside C, Hershberg, Wolfe, and Fieser (9) report that the cardiotonic glycosides and their genins fall within the scope of the polarographic method of determination. Karrer (10) has announced that the fractionation of digalene appears to yield two new glycosides, one of which has approximately the same potency as digitoxin, while the other has a potency almost 50 per cent higher than that of digitoxin. He has also announced the isolation of a new glycoside, diginin, which is practically inactive as a cardiotonic but gives a positive Legal's reaction.³ Hagemeyer (11) reports that diginin shows a high color activity with alkaline picric acid, and Shoppee and Reichstein (12) have studied the chemistry of this substance. Quite obviously this glycoside is of importance to our studies and we have planned an examination of its reactivity toward the Baljet reagent.

SUMMARY

1. The relative intensities of the color reaction of solutions of seven digitalis glycosides toward alkaline picrate solutions have been determined. These glycosides are lanatosides A, B, and C, digoxin, digitoxin, gitoxin, and gitalin.
2. Confirming earlier work, the color intensities produced by the *purpurea* glycosides parallel the cardiotonic activity. This relationship does not hold in the case of the *lanata* glycosides and therefore, mixtures of these glycosides cannot be assayed by this color intensity.
3. An explanation has been offered to account for the differences in reactivity among the *purpurea* glycosides and digoxin based upon the supposition of the formation of isomers which are inactive. On the same basis, an explanation has been given for the observed divergence from Beer's law of the Baljet color reaction with these glycosides.

³ It is of interest to note that W. A. Jacobs (Physiol. Rev. 13: 222, 1933) reported a glycoside allocymarin in which cymarin had been isomerised to a compound which gave the Baljet reaction but was quite devoid of cardioactivity. Other similar instances have been recorded.

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STUDIES ON THE OCULAR REACTIONS OF RABBITS TO DI-ISOPROPYL FLUOROPHOSPHATE¹

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The investigations reported here were undertaken to determine the effects of di-isopropyl fluorophosphates on rabbits' eyes. Since many of the expected ocular reactions, such as visual disturbances, and pain, can be studied satisfactorily only on man, the present experiments were planned as preliminary to such a study.

MATERIALS AND METHODS. Young adult mongrel rabbits were used. Vapor applications were produced with the single eye vapor chamber in use in this laboratory (1). Single drop application of di-isopropyl fluorophosphate, either pure or diluted with triacetin or propylene glycol, were made from a standard eye dropper or measured and delivered directly to the eye from a microsyringe.

RESULTS. Table 1 shows the results of the exposure of rabbits' eyes to the saturated vapor of di-isopropyl fluorophosphate at 24°C. Short exposures, 15 seconds, produce a maximal miosis just as soon as longer exposure. Themiosis disappears more quickly after the 15 second exposure, the pupil being normal after 24 hours, while after a 1 minute exposure the miosis disappears in 24 hours but the pupil remains sluggish in its reaction to light for another 24 hours. An exposure of 2 minutes results in a sluggish pupil for 2 days. Except for some mild conjunctival congestion and a transient rise of intraocular pressure immediately after prolonged exposure, no other clinical signs appear even if the animals are observed for several weeks.

No cumulative effects were noted when eyes were exposed for 30 seconds every other day for one week (table 2). Recovery of the pupillary signs took place 24 hours after the last exposure just as in the case of eyes receiving one exposure.

The instillation into the cul-de-sac of single drops of the diluted drug is followed by marked lacrimation and then by rapid pupillary constriction. The rate of constriction depends upon the concentration of the drug (table 3). No cumulative reactions were found on repeated applications.

When the undiluted drug was instilled directly into rabbits' eyes in an attempt to produce permanent damage, it was found to be extremely toxic. Rabbits receiving 1.4 mgm. per kilogram (table 4) by this route of administration, died. No ocular changes other than those noted above were found even with these doses.

When lethal doses were applied to the eyes of rabbits under ether anesthesia, marked weakness and paralysis of the hind legs appeared at 5 minutes following the recovery from anesthesia. At 7 minutes violent generalized convulsions,

¹ The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

TABLE 1

Single exposure of rabbit eyes to di-isopropyl fluorophosphate vapor

NO. OF EYES	SECONDS EXPOSURE TO SATURATED VAPOR AT 24°	AVERAGE APPEARANCE OF MAXIMAL MIOSIS MINUTES AFTER EXPOSURE	PUPILLARY REACTIONS		
			1 day	2 days	3 days
2	15	4	normal		
17	30	4	sluggish	normal	
1	60	4	sluggish	normal	
5	120	4	sluggish	sluggish	normal

TABLE 2

Repeated exposure of rabbits' eyes to di-isopropyl fluorophosphate vapor
 5 eyes exposed for 30 seconds to saturated vapor at 24° every other day

DAYS	EXPOSURE	PUPILLARY REACTION BEFORE EXPOSURE
1	+	normal
2	0	sluggish
3	+	normal
4	0	sluggish
5	+	normal
6	0	sluggish
7	+	normal
8	0	sluggish
9	0	normal

TABLE 3

Instillation of 2 drops (approximately 0.1 cc.) of solutions of di-isopropyl fluorophosphate into rabbits' eyes

NUMBER OF EYES	CONCENTRATION	SOLVENT	AVERAGE APPEARANCE OF MAXIMAL MIOSIS MINUTES AFTER INSTILLATION	REMARKS
2	%			
2	0.015	triacetin	25	sluggish pupillary reactions for 12-24 hours, then return to normal
1	0.06	triacetin	15	
3	0.25	triacetin	9	
4	0.5	triacetin	7	
4	0.5	propylene glycol	20	
5	1.0	propylene glycol	7	sluggish pupillary reaction for 24-48 hours, then normal
5	1.0	triacetin	7	
2	1.0	propylene glycol	7	dose repeated every other day for seven days—complete recovery 48 hours after last dose

miosis, lacrimation and occasional diarrhea set in. These signs continued with increased severity until respiration ceased following which there would be isolated

twitchings of the muscles for several minutes. Animals that survived for 24 hours were weak and exhibited numerous muscle twitchings until death ensued, apparently from general exhaustion.

Because of previous reports (2) of increased intraocular tension, absolute and relative measurements of the intraocular tension were made with the Friedenwald manometer (3) and Schiotz tonometer. Coincidental with the constriction

TABLE 4

Local application of undiluted di-isopropyl fluorophosphate to rabbits' eyes

ANIMAL NO.	DOSE APPLIED TO EYES	TIME OF DEATH AFTER APPLICATION	REMARKS
	<i>mgm./kg.</i>		
S 931	1.15	survived	no permanent eye lesion, pupil normal in 2 days pupil sluggish
S 938	1.4	24 hours	
S 939	1.4	30 minutes	
S 937	2.8	4 hours	
S 934	2.8	before 24 hours	
S 933	5.7	5 minutes	
S 936	5.7	10 minutes	
S 932	8.5	3 minutes	

TABLE 5

Effect of atropine on pupillary signs of rabbits exposed to di-isopropyl fluorophosphate vapor

NO. OF ANIMALS	EXPOSURE TO SATURATED VAPOR OF DI-ISOPROPYL FLUOROPHOSPHATE AT 24°	TIME OF APPLICATION OF ATROPINE 2%, 4 DROPS	REMARKS
	<i>seconds</i>		
3	30	25 min. before exposure	atropinized pupil did not constrict. Controls constricted in 4 min.
1	30	Immediately after exposure	delayed slightly, but did not prevent maximal constriction
3	30	24 hours after exposure when pupil is still sluggish	pupils dilated and remained so

of the pupil, the tension rises about 15 to 30 millimeters of mercury and begins to return to normal again about 15 minutes later.

Histologic examination of eyes removed during the period of elevated tension showed epithelial blisters on the ciliary processes, engorgement of the iris and ciliary vessels, coagulated protein in the posterior and anterior chambers.

The eserine-like action of di-isopropyl fluorophosphate prompted studies of the local protective effect of atropine on animals exposed to the drug. Table 5 shows that atropine applied before the application of the vapor was able to pre-

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THE TREATMENT OF DI-ISOPROPYL FLUOROPHOSPHATE (DFP) POISONING IN RABBITS

BERNARD P. McNAMARA,¹ GEORGE B. KOELLE² AND ALFRED GILMAN³

From the Pharmacology Section, Medical Division, Edgewood Arsenal, Maryland

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Physiological effects of dimethyl and diethyl fluorophosphate were noted in 1932 by Lange and Krueger (1), who first prepared these esters. In 1940, McCombie (2) et al. re-investigated these compounds and found them to be lethal inhalants of high toxicity. It was suggested in a subsequent paper (3) that the sudden death produced by effective concentrations, and the acute miosis and loss of accommodation caused by low concentrations, made the alkyl fluorophosphates worthy of investigation as offensive gases.

Homologues were prepared for comparison (4) and di-isopropyl fluorophosphate (DFP) was found to possess the maximum toxicity of the group.

Similarities between the action of the fluorophosphates and eserine evoked the suggestion that the former esters might possess anticholinesterase activity. Mackworth (5) investigated this possibility and concluded that the fluorophosphates did inhibit cholinesterase. However, unlike eserine, the inhibitory effect on the enzyme could not be reversed by dialysis.

The mechanism of action indicated atropine as an antagonist against the physiological effects of the fluorophosphates. Barrett et al. (6) reported that atropine even in large doses given immediately after di-isopropyl fluorophosphate was incapable of saving life though it did alleviate symptoms and prolong life. They further stated that when given before the ester it delayed the development of symptoms and reduced their severity, and suggested that repeated doses might prevent death.

The Toxicity Laboratory of the University of Chicago (7) reported that the protective action of atropine or acetylcholine against the effects of dimethyl fluorophosphate was questionable, whereas mecholyl offered appreciable protection in mice. This protection was explained on the basis that mecholyl decreased the respiratory intake and thus limited the amount of dimethyl fluorophosphate absorbed.

Anticholinesterase drugs are toxic by virtue of both nicotinic and muscarinic actions. No single drug is known which is capable of blocking both these effects. Atropine is highly parasympatholytic in clinical doses. However most agents which block the nicotinic actions are more difficult to control. In fact experimental attempts to mask the nicotinic actions of DFP with curare were unsuccessful. Inasmuch as magnesium sulfate, which possesses a curare-like action, has been widely employed clinically, it seemed a possible choice as an agent to

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vent the onset of signs. If applied immediately after the toxic agent, a slight delay in the onset of maximum pupillary constriction was achieved. Twenty-four hours after the application of the vapor, the sluggish pupil could be easily dilated with atropine.

DISCUSSION. When lethal doses of di-isopropyl fluorophosphate are applied topically to the eyes, death is of the same character as caused by eserine poisoning with rapid onset of miosis, lacrimation, fine muscular twitchings, coarse convulsions, salivation and occasional diarrhea. Death follows apparent paralysis of respiration and there are some fine muscular twitchings for several minutes post-mortem. This series of signs coupled with the information that the drug inhibits cholinesterase gives strong evidence that the main symptoms of the poisoning are due to an excess of acetylcholine.

The ocular signs of miosis and lacrimation which follow local application represent the muscarine-like action of acetylcholine. These signs can be blocked by a prior local administration of atropine or, if the signs are already established, they can be reversed with atropine. A similar reversal has been accomplished in man poisoned with di-isopropyl fluorophosphate (5). Because the rabbit destroys atropine quite readily, it is not a good experimental animal for these experiments and further studies on the ocular effect of di-isopropyl fluorophosphate are to be made on man.

SUMMARY

1. No permanent ocular lesions were found in rabbits when an almost lethal dose or repeated small doses were applied to the eyes.

2. Local application to rabbits' eyes of di-isopropyl fluorophosphate vapor or solution causes intense miosis, lacrimation, transient increase in intraocular pressure.

3. 1.4 mgm./kg. applied to a rabbit's eye is lethal.

4. Atropine applied before di-isopropyl fluorophosphate prevents the development of the miosis; applied after the drug, it relaxes pupillary contraction.

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Anticholinesterase drugs are toxic by virtue of both nicotinic and muscarinic actions. No single drug is known which is capable of blocking both these effects. Atropine is highly parasympatholytic in clinical doses. However most agents which block the nicotinic actions are more difficult to control. In fact experimental attempts to mask the nicotinic actions of DFP with curare were unsuccessful. Inasmuch as magnesium sulfate, which possesses a curare-like action, has been widely employed clinically, it seemed a possible choice as an agent to

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block the nicotinic actions of DFP. Combination therapy with magnesium and atropine would theoretically antagonize the two major actions produced by this agent.

EXPERIMENTAL. *Toxicity of DFP after parenteral administration in rabbits.* The results obtained when solutions of DFP freshly dissolved in propylene glycol were administered intravenously and intramuscularly to rabbits are summarized in table 1.

The outstanding symptoms exhibited by these animals were those resulting from the nicotinic actions of acetylcholine. Muscle tremors and spasticity of the hind legs could be observed within one minute after the intravenous administrations of 0.5 mgm./kgm. The animals rapidly lost fine control of their muscles and became ataxic. Nicotinic stimulation was soon followed by muscular paralysis, first noticeable in the neck and forelegs and eventually extending to the hind legs. The animals also showed central stimulation evidenced by excitement and convulsions. Death appeared to result from respiratory paralysis. With lower doses, the above symptoms were prolonged but followed the same relative course.

TABLE 1
Intravenous and intramuscular toxicity of DFP in rabbits

ROUTE	DOSAGE	DEATH	TIME OF MAJORITY OF DEATHS
	mgm./kgm.		
Intravenous.....	0.3	2/10	3½-4 hours
Intravenous.....	0.4	10/12	14 min.-2 hours
Intravenous.....	0.5	10/10	5 min.-21 min.
Intramuscular.....	0.5	0/2	
Intramuscular.....	0.75	1/2	83 min.
Intramuscular.....	1.0	7/7	47 min.-4½ hours

Two animals receiving 0.4 mgm./kgm. survived 3 and 7 days respectively, the paralysis of the neck and leg muscles persisting until death.

The muscarinic symptoms were mild as compared to the nicotinic effects, even in animals surviving for several hours. Many of the animals failed to show miosis, very few salivated and there was little evidence of increased motor activity of the gastro-intestinal tract. In view of the fact that the dosage of DFP employed was sufficient to reduce markedly the concentration of tissue cholinesterase (8) the mild nature of the muscarinic responses seems an anomaly. It can best be explained by presuming that the nicotinic stimulation of sympathetic ganglia and the adrenal medulla effectively antagonized the peripheral muscarinic actions.

The effect of prophylactic barbiturate anesthesia. Pentobarbital anesthesia (25.0 mgm./kgm.) afforded rabbits no appreciable protection from the lethal action of DFP. However, it did alter the clinical picture. Similar results have been reported by Friedenwald (9). In the anesthetized animals convulsions were not evident but muscle tremors were persistent. In contrast to the unanesthetized rabbits, muscarinic effects were marked. The animals exhibited pin point pupils,

bronchorrhea, copious diarrhea and profuse salivation. Nine of the ten animals previously treated with pentobarbital died after the intravenous administration of 0.5 mgm./kgm. of DFP. Death, however, was significantly delayed, occurring in from one to 19 hours. The one animal which survived exhibited muscular weakness for a period of five days. The unmasking of marked muscarinic actions by pentobarbital anesthesia, in keeping with the explanation offered above, may presumably have been due to the depressant action of barbiturates on autonomic ganglia and the adrenal medulla.

Prophylactic effect of barbiturate anesthesia and atropine. Combination therapy with large doses of atropine sulfate (5.0 mgm./kgm. intravenously or 20.0 mgm./kgm. intramuscularly) and pentobarbital was attempted in view of the fact that the muscarinic symptoms of DFP were so prominent in the anesthetized animals. The only symptoms exhibited by these animals following the intravenous injection of 0.5 mgm./kgm. of DFP was a persistent muscle tremor. Of 10 animals so treated, 6 died between 18 hours and 6 days, and 4 survived.

Prophylactic treatment with atropine alone failed to save any of 4 rabbits, although death was delayed for approximately 2 hours. It should be pointed out that at this high dose level atropine may also exert a blocking action against the nicotinic effects of DFP.

Prophylactic treatment with magnesium sulfate and atropine. Combined prophylactic treatment with magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and atropine sulfate prevented almost completely all symptoms following the intravenous injection of 0.5 mgm./kgm. of DFP for varying periods. For example, prophylactic treatment by the administration of 200 mgm./kgm. of magnesium sulfate and 5.0 mgm./kgm. of atropine sulfate intravenously maintained animals symptom free for a period of 30 to 60 minutes, following which symptoms of reduced severity appeared. Protection was afforded despite the fact that this dose of magnesium produced little depression. Three of 6 animals receiving 0.5 mgm./kgm. of DFP intravenously recovered with this treatment as did all of 3 animals receiving 0.4 mgm./kgm. of DFP intravenously.

Intramuscular prophylaxis with larger doses, 800 mgm./kgm. of magnesium sulfate with 20 mgm./kgm. of atropine sulfate, protected animals from the toxic actions of DFP for longer intervals. However, at this dose level magnesium therapy caused marked depression. Nine of 14 animals receiving this type of prophylactic therapy survived the effects of 0.5 mgm./kgm. of DFP given intravenously and 2 of 2 animals survived the effects of 0.4 mgm./kgm.

Magnesium sulfate alone in the prophylactic intramuscular dose of 800 mgm./kgm. saved only 2 of 13 animals, indicating the importance of blocking muscarinic effects. The muscarinic actions were not as severe in the animals receiving magnesium sulfate as in those receiving pentobarbital. Two animals receiving prophylactic treatment with 800 mgm./kgm. of magnesium sulfate intramuscularly, survived the effects of 0.4 mgm./kgm. of DFP.

Prophylactic treatment with magnesium sulfate and acetyl-beta-methyl-choline. The University of Chicago Toxicity Laboratory (7) has reported protection from the lethal effects of DFP with large doses of acetyl-beta-methyl-choline. In the

present study, when acetyl-beta-methyl-choline was given in addition to magnesium sulfate and atropine, the animals seldom showed any symptoms from the effects of DFP as long as effective concentrations of magnesium were maintained. However, the symptoms recurred and only 2 of 9 animals survived the effects of 0.5 mgm./kgm. of DFP intravenously following prophylactic intravenous therapy with 200 mgm./kgm. magnesium sulfate, 5.0 mgm./kgm. atropine sulfate and 5.0 mgm./kgm. acetyl-beta-methyl-choline. On the other hand, when this combination was given intramuscularly in dosage of 5.0 mgm./kgm. of atropine sulfate and 800 mgm./kgm. of magnesium sulfate, 20 mgm./kgm. of acetyl-beta-methyl-choline prior to the intravenous administration of 0.5 mgm./kgm. of DFP, 5 of 5 animals recovered.

A possible explanation for the protective action of acetyl-beta-methyl-choline is that it combines with the cell receptors thereby denying this site to acetylcholine. Another possibility is that it protects the enzyme from combination with DFP. The results with various types of prophylactic treatment are presented in table 2.

Delayed treatment with magnesium sulfate and atropine. Magnesium sulfate and atropine have proven effective therapeutic agents when administered after the systemic effects of DFP have become manifest. For studies of this type it was necessary to administer DFP by intramuscular injection in order that time might be available for effective treatment. Following the intramuscular injection of 1.0 mgm./kgm. of DFP (LD_{100}) symptoms are apparent within 10 minutes and death occurs in from 15 minutes to $4\frac{1}{2}$ hours. Of 3 groups of 2 animals each, treated 15, 30 and 45 minutes respectively after the intramuscular administration of 1.0 mgm./kgm. of DFP, all recovered. Treatment consisted of the intramuscular injection of 400.0 mgm./kgm. of magnesium sulfate and 10 mgm./kgm. of atropine sulfate at the indicated time followed by smaller doses of this combination when needed. Therapy was usually effective as long as symptoms had not progressed to the stage of flaccid paralysis, in the event of which even the cautious administration of magnesium sulfate was apt to precipitate death.

Prophylactic and delayed treatment of DFP vapor poisoning in rabbits. In the preceding experiments where DFP was given parenterally, magnesium sulfate effectively blocked the nicotinic actions and atropine prevented the muscarinic effects. However, it was necessary to test the therapeutic action of this drug combination following vapor exposure since in this type of exposure high concentrations of DFP can act directly on the respiratory tract. It is not unreasonable to presume under these circumstances that death might result from a marked muscarinic action on the bronchial musculature and secretory mechanism.

Rabbits were gassed in a 629 liter dynamic chamber using a flow rate of 250 liters/minute. The DFP was introduced into the chamber by bubbling a controlled stream of nitrogen through liquid DFP heated to 60°C . Concentrations were determined by collecting samples of the chamber air in 5.0% sodium hydroxide, neutralizing, digesting with H_2SO_4 and analyzing for phosphorous by the Fiske-Subbarow (10) method. Standard solutions of DFP yielded theoretical amounts of phosphorous by this analytical procedure.

In preliminary experiments it was found that a concentration of 1150 gamma/l. for a period of 7 minutes (CT = 8050) killed a majority of the animals and yet allowed sufficient time for treatment after removal of the animals from the chamber. In the various experiments analytical CT values ranged between 7600 and 8800. In all instances experimental and control animals were exposed at the same time so that the unavoidable variation in concentration in no way influenced the interpretation of results.

TABLE 2
Treatment of poisoning produced by DFP in rabbits

DFP	PROPHYLACTIC AGENT	DEATHS
Prophylaxis		
mgm./kgm. 0.50 i.v.	Nembutal (25.0 mgm./kgm., i.v.)	9/10
0.50	Nembutal (25.0 mgm./kgm., i.v.) Atropine (20.0 mgm./kgm., i.m.)	6/10
0.50	Atropine	4/4
0.50	MgSO ₄ (200.0 mgm./kgm., i.v.) Atropine (5.0 mgm./kgm., i.v.)	3/6
0.50	MgSO ₄ (800.0 mgm./kgm., i.m.)	11/13
0.50	MgSO ₄ (800.0 mgm./kgm., i.m.) Atropine (20.0 mgm./kgm., i.m.)	5/14
0.50	MgSO ₄ (200.0 mgm./kgm., i.v.) Atropine (5.0 mgm./kgm., i.v.) Mecholyl (5.0 mgm./kgm., i.v.)	7/9
0.50	MgSO ₄ (800.0 mgm./kgm., i.m.) Atropine (20.0 mgm./kgm., i.m.) Mecholyl (20.0 mgm./kgm., i.m.)	0/5
Treatment		
1.0 i.m.	MgSO ₄ (400.0 mgm./kgm., i.m.) Atropine (10.0 mgm./kgm., i.m.)	0/6

Four types of experiments were performed: 1) prophylactic treatment with magnesium sulfate and atropine sulfate; 2) treatment with magnesium sulfate and atropine sulfate after exposure; 3) prophylactic treatment with atropine sulfate alone; 4) treatment with atropine sulfate alone after exposure. The magnesium sulfate (400 mgm./kgm.) and atropine sulfate (10 mgm./kgm.) were administered intramuscularly. Prophylactic treatment was given 15-30 minutes before exposure and post-exposure therapy was instituted immediately after the

animals were removed from the chamber. An occasional animal was given a second dose of magnesium sulfate and atropine sulfate if symptoms were not adequately controlled by the first. The results obtained are presented in table 3.

It is apparent from table 3 that combined treatment with magnesium sulfate and atropine sulfate is a very effective form of therapy for combating vapor exposures to DFP. Of special interest is the fact that prophylactic treatment with magnesium sulfate and atropine sulfate is less effective than therapy that is instituted after exposure. This apparent anomaly is probably the result of the protective respiratory reflexes which operate in untreated animals. The species resistance of rabbits to dimethyl fluorophosphate vapor has been emphasized by the Chicago Toxicity Laboratory (11). This resistance is lost when vapor is introduced directly into the trachea in which event protective reflexes reducing respiratory minute volume are not operative. It is felt that in the above experiments the sedative action of magnesium sulfate had affected the animals receiving prophylactic treatment in such a way as to permit the absorption of larger amounts of DFP than the controls.

TABLE 3

Treatment with magnesium sulfate and atropine sulfate in rabbits following exposure to an analytical CT of 7,600-8,800 of DFP*

Exposure time—7 minutes

	CONTROLS	MgSO ₄ AND ATROPINE SULFATE		ATROPINE SULFATE	
		Prophylactic	Therapeutic	Prophylactic	Therapeutic
Number of deaths.....	38/46	7/15	2/15	8/15	8/15
Percentage deaths.....	83	47	13	53	53

* CT—Concentration in micrograms/liter X time in minutes.

DISCUSSION. From the foregoing experiments it can be seen that DFP exerted both nicotinic and muscarinic actions. Atropine, by virtue of its parasympatholytic action effectively blocked the muscarinic effects. Animals thus treated exhibited the muscle paralysis and tremors caused by nicotinic stimulation but showed none of the miosis, salivation, increased gastro-intestinal motility or diarrhea associated with the muscarinic effects. However, when magnesium sulfate was used by itself, the opposite was true. Here the nicotinic effects were blocked and the skeletal muscle stimulation did not occur. On the other hand, all of the muscarinic effects mentioned above became emphasized. When both systems were blocked, a maximum protection was obtained as shown by the data.

Pentobarbital blocked neither the muscarinic nor the nicotinic actions and marked symptoms associated with these effects were seen. The little protection which it did offer was conceivably due to its sedative action with a resulting decrease in acetylcholine liberation.

In view of the established clinical background of both magnesium sulfate and atropine it is felt that these drugs can be used against poisoning produced by DFP. Since broncho-constriction may be an alarming symptom in humans, the intra-

muscular administration of epinephrine hydrochloride or the intravenous administration of aminophylline may prove necessary.

CONCLUSIONS

1. For effective treatment of poisoning produced by DFP it was necessary to block both the muscarinic and nicotinic effects.

2. Atropine sulfate prevented the muscarinic effects produced by DFP but was of little value when used alone since moderate doses did not block the nicotinic effects.

3. Pentobarbital, when used alone, prevented neither the muscarinic nor the nicotinic effects and was of little value as a therapeutic agent although it did prolong the survival time.

4. Magnesium sulfate prevented the nicotinic effects of DFP but offered poor protection when used alone.

5. The combination of atropine sulfate and pentobarbital was only slightly better than atropine alone and did not protect rabbits from a nicotinic death.

6. The combination of atropine sulfate and magnesium sulfate prevented both the muscarinic and the nicotinic effects of DFP and afforded the best protection of any method of treatment tried.

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ANTIDOTES TO POISONING BY DI-ISOPROPYL FLUOROPHOSPHATE IN CATS¹

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The observation by Mackworth (1) which has been amply corroborated by others, to the effect that fluorophosphates inhibit cholinesterase, satisfactorily explains the "muscarinic" effects of these compounds. A more complete analysis of the effects of di-isopropyl fluorophosphate (DFP) shows, however, that both "muscarinic" and "nicotinic" actions are involved in rabbits (2) and in cats (3). The type of antidotes which might prove effective against DFP would depend upon which of these mechanisms was responsible for the lethal effects of DFP. Barrett, Feldberg, Kilby, and Kilby (4) found that atropine lessened symptoms and prolonged life in the rabbit, but failed to save the animals. A study made in the Toxicity Laboratory of the University of Chicago (5) also reported atropine of questionable protective value against DFP in mice. The subject was further investigated by McNamara, Koelle, and Gilman (2) in rabbits. They found atropine or magnesium alone of little value, but the combination in suitable doses exerted a distinct influence in preventing symptoms and increasing the incidence of survival. Such a combination enabled rabbits to recover from a dose of DFP which was twice that from which untreated rabbits almost regularly died.

There are species differences in the reactions to DFP. The cat is about 5 times as tolerant as the rabbit to DFP (6); the intravenous LD₅₀ for the cat, 1.6 mg. per kg. and for the rabbit, 0.34 mg. per kg. There is also the observation that the "muscarinic" actions which are fairly prominent in the cat (3), are overshadowed by the "nicotinic" actions in the rabbit (2). These suggest the possibility that the problem of antidotes may be different in different species.

The present study was carried out to explore the value of atropine, magnesium, and other metals as antidotes to DFP in the cat.

EXPERIMENTAL. The antidotes were studied with respect to their influence on 1) the transient symptoms of DFP poisoning, 2) the survival rate after DFP, and 3) the protracted symptoms of DFP poisoning. The use of these agents for prophylaxis before DFP, and for treatment after DFP was explored.

The experiments involving atropine and magnesium fell into 4 groups:

1. DFP alone (27 cats)
2. Atropine before or after DFP (69 cats)
3. Magnesium before DFP (15 cats)
4. Combination of magnesium and atropine before DFP (27 cats).

Solutions. The DFP was made up in the form of a 1 per cent solution in physiological saline and used within 2 hours or less. It was always injected intravenously. The atropine

¹ The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Cornell University Medical College.

² This study is part of a cooperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the name of the chief collaborators.

sulfate was used in the form of a 0.1 to 1.0 per cent solution in physiological saline, and given either intravenously or intramuscularly. The magnesium sulfate, U.S.P., was used in the form of a 50 per cent solution in water and injected intramuscularly. All doses in this report are expressed in terms of mg. per kg. of body weight.

RESULTS. *Protective effect of atropine alone.* The results are summarized in table 1. Atropine exerted a strong protective effect when given either before or as long as 30 minutes after the DFP.

Some of the symptoms of DFP poisoning, namely, unrest, rage, salivation, pilomotor stimulation and convulsions were promptly suppressed or terminated

TABLE 1
Effect of atropine on the course of DFP poisoning

NO. OF CATS	DOSE DFP (VEIN)	ATROPINE		PER CENT MORTALITY	AVERAGE TIME TO DEATH	AVERAGE TIME TO RECOVERY
		Dose (vein)	When given			
	mg./kg.	mg./kg.				
19	3.0	none		63	23 hrs.	66 hrs.
10	3.0	0.1	15 min. before	0		78 hrs.
6	3.0	1.0*	15 min. before	0		65 hrs.
8	3.0	1.0	6 min. after	12.5	96 hrs.	4 days
5	3.0	1.0	15 min. after	0		5 days
5	3.0	1.0	30 min. after	20	18 min.	8 days
8	5.0	none		100	30 min.	
4	5.0	0.1	20 min. before	100	4 hrs.	
8	5.0	1.0	20 min. before	25	7 hrs.	6 days
8	5.0	1.0	5 min. after	37.5†	27 min.	9 days
4	7.0	1.0	5 min. before	75	22 min.	?
7	7.0	10.0	5 min. before	14‡	25 min.	10 days
1	10.0	20.0	5 min. before	100	10 min.	
3	10.0	10.0	5 min. before	100	19 min.	

* Intramuscular injection.

† Does not include 3 cats which died of pneumonia after apparently recovering from the DFP.

‡ Does not include 1 cat which died of pneumonia on the 8th day.

by doses of atropine varying from 0.1 to 20 mg. This applied to the whole range of DFP dosage used in these experiments, namely, from 3 mg. (LD63) to 3.3 times as much. It applied to animals which succumbed as well as to those which survived. The difference between the appearance of the protected and unprotected animal was striking. In some protected animals the only visible effect of the DFP was generalized muscular twitching; in others the twitching was combined with weakness and prostration.

Atropine in the foregoing doses also enabled cats to survive otherwise fatal doses of DFP. The protective action was very high in the group of animals receiving the LD63 dose of DFP. All animals survived this dose when previously

given atropine. The protection was only slightly less when the atropine was given between 6 and 30 minutes after the DFP.

The protective influence of atropine was still quite marked in the case of the LD100 and 1.4 times the LD100 dose of DFP (5 and 7 mg.). After atropine, 86 per cent of the cats survived the latter dose of DFP.

The limit of protection by atropine against a fatal outcome seems to be reached at a dose short of 10 mg. of DFP or about 2 times the LD100.

It may be noted that there is a relationship between the dose of DFP and the dose of atropine which is necessary to insure survival. For example, whereas only 25 per cent of the animals succumbed in the 5 mg.-group when treated with 1 mg. of atropine, 75 per cent succumbed in the 7 mg.-group when treated with the same dose of atropine, but only 14 per cent succumbed in the 7 mg.-group when the dose of atropine was raised to 10 mg.

Animals which, as the result of protection by atropine survived otherwise fatal doses of DFP, regularly showed an effect rarely encountered after the smaller doses of DFP (animals surviving without protection), namely, protracted signs of poisoning extending over a period as long as about two weeks. During this period the animal might appear sleek, well groomed and otherwise normal when sitting quietly, but when placed on its feet and urged to walk, signs of severe myoneural dysfunction became apparent. They walked with difficulty. There was weakness, especially of the hind limbs. After only a few steps they fell over and could not be provoked into walking even by a painful stimulus although they responded by crying. Some grew progressively worse and died in about 2 days, or as in the case of most of them, the condition improved and disappeared completely in a week or two. Sustained pilomotor stimulation was also seen in these animals and often persisted for a week or longer after the animal was otherwise normal. The tail, especially at the base, resembled that of a squirrel. The pilomotor effect could usually be obtained by exciting the animal.

Protective effect of magnesium alone. The results are summarized in table 2. The magnesium was administered in doses of 250 and 500 mg. before the DFP. The smaller dose of magnesium frequently produced no effects, or drowsiness, the larger dose usually produced anesthesia. The magnesium regularly suppressed the fibrillary twitchings of DFP poisoning (doses, 3 to 7 mg.); feeble fibrillations often returning after several hours. The unrest and excitement were suppressed in the cases in which the animal was narcotized by the magnesium, otherwise those symptoms of DFP were present as in the unprotected animal.

Magnesium increased the survival rate but to a very much smaller degree than atropine. The LD63 dose of DFP was reduced to an LD17. There was no influence on the survival rate following larger doses of DFP. The limited protective effect of magnesium against the fatal action of DFP did not appear to be related to a state of narcosis since the dose in this group was 250 mg. and after this dose the animal usually appeared normal.

Protective effect of the combination of magnesium and atropine. In these experiments the atropine was given intravenously and the magnesium intramus-

cularly with an interval of approximately 10 minutes or less between them, and these were followed, usually within about half an hour, by the dose of DFP. The combination of magnesium and atropine was a more effective prophylactic than either of the two drugs alone. The contribution of magnesium was greater than one might infer from the results of magnesium alone. Whereas its influence as the sole antidote was not in evidence beyond the level of the 3 mg. dose of DFP, in the combination its influence was fairly strong throughout the whole range of doses of DFP that were used, namely, from 5 to 10 mg. At the 5 mg. dose of DFP the combination with magnesium eliminated the 25 per cent mortality present when atropine was the sole protective agent. A similar contribution was in evidence at the 7 mg. dose level. The ceiling protective action

TABLE 2
Effect of magnesium on the course of DFP poisoning

NO. OF CATS	DOSE DFP (VEIN)	DOSE ATROPINE (VEIN)	DOSE MgSO ₄ (muscle)	PER CENT MORTALITY	AVERAGE TIME TO DEATH	AVERAGE TIME TO RECOVERY
	mg./kg.	mg./kg.	mg./kg.			
19	3.0	0.1-1.0	250	63	23 hours	66 hours
16	3.0			0		73 hours
6	3.0			17	72 hours	77 hours
8	5.0	1.0	250-700	100	30 min.	6 days
8	5.0			25	7 hours	
4	5.0			100	18 min.	
6	5.0	1.0		0		5 days
4	7.0	1.0	500	75	22 min.	10 days
7	7.0	10.0		14	25 min.	
5	7.0			100	29 min.	
4	7.0	1.0	500	25	6 days	10 days+ 6 days+
3	7.0	0.1	500	33	32 min.	
4	7.0	0.1	250	100	10 hrs.	
4	10.0	10.0-20.0	500	100	17 min.	13 days+
10	10.0	10.0		60	(4)-1 hr. (2)-72 hrs.	

of atropine was raised; at the 10 mg. dose of DFP at which atropine provides no survivals, the combination with magnesium brought about a survival of 40 per cent of the animals. There is also some indication that in the presence of magnesium ineffectual doses of atropine became effective antidotes, and further that an optimum dosage ratio of magnesium to atropine may exist. These points require further study with larger numbers of animals.

The transient symptoms following DFP were completely abolished by the combination; atropine preventing the rage, unrest, salivation and pilomotor effects, and the magnesium suppressing fibrillary twitchings. The combination, however, had no influence on the protracted symptoms of poisoning following the large doses of DFP such as have been discussed in relation to the atropine alone.

DISCUSSION. The foregoing results in the cat are in some respects similar to those of McNamara, Koelle, and Gilman (2) in the rabbit. They show that atropine suppresses the "muscarinic" actions of DFP and magnesium the "nicotinic" actions, and that the combination of the two is more effective than either alone. They differ from the observations in the rabbit, however, in that atropine alone shows a very strong protective action against DFP, enabling animals to survive about twice the average lethal dose. The protective action of magnesium alone is relatively slight, however, when magnesium is combined with atropine, the protective action of magnesium becomes quite conspicuous.

The facts brought to light in the present report fall into line if one postulates that the cause of death following moderate doses of DFP is chiefly the action antagonized by atropine (the "muscarinic" action), and that within a limited range of doses of DFP this action is sufficient by itself to cause death. When the dose of DFP is very large, however, an action appears which may cause death and is not blocked by atropine. McNamara, Koelle and Gilman (2) refer to this as the "nicotinic" lethal action, antagonized by magnesium. It is clear enough that magnesium prevents the fibrillary twitchings caused by DFP, but it is not quite clear how such an action would cause death nor how its prevention by magnesium saves the animal's life. The possibility needs to be considered that magnesium supplements the protective action of atropine by a central depression. There is also the possibility of some chemical interaction between DFP and magnesium. The latter received some support from exploratory experiments with other metals which showed that salts of calcium, aluminum, and gold, but not of strontium, exerted some protective action against DFP, and that this protective action did not appear to be related to the capacity of the metal to relieve fibrillary twitching. The subject is in need of further study.

SUMMARY

1. In cats atropine given before or after DFP abolished the "muscarinic" symptoms, reduces or abolishes the central excitant effects, and provides marked protection against the fatal action of DFP.
2. Magnesium controls the fibrillary twitches of skeletal muscle caused by DFP, but alone affords but slight protection against the fatal action of DFP.
3. A considerable further degree of protection against DFP is afforded by the simultaneous use of magnesium and atropine.
4. The results indicate that DFP causes death by two mechanisms, one of which is blocked by atropine, the other brought on by larger doses is blocked by magnesium.

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SYNERGISMS AND ANTAGONISMS BETWEEN PHYSOSTIGMINE AND DI-ISOPROPYL FLUOROPHOSPHATE IN CATS¹

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In the course of a study on the pharmacologic relationships between diisopropyl fluorophosphate (DFP) and other drugs influencing structures innervated by the autonomic nervous system, it was observed that physostigmine exerts a marked protective action against the toxic effects of subsequent doses of DFP, but when given after DFP the opposite is true, i.e., not only is there no protection, but the toxic effects of physostigmine are enhanced. Because of this interesting relationship between two substances, both of which have as their chief action the inhibition of cholinesterase, the phenomenon has been studied in some detail.

Toxicity of physostigmine. In a total of 69 cats given various doses of physostigmine by vein, the calculated value of the LD 50 was 0.66 mg.³ The detailed data are given in table 1. 15 of 16 cats died from 1 mg. (LD 94) and 5 of 24 (LD 21) from 0.5 mg. In general the severity of the toxic symptoms and the time to death were proportional to the dosage although individual animals showed considerable variability. Mild symptoms were present following 0.1 to 0.25 mg., but there was none from 0.01 or 0.05 mg.

Tolerance to physostigmine. In view of the protection afforded by physostigmine against the toxic action of DFP described below, a number of animals was tested with repeated doses of physostigmine given intravenously in order to determine whether mere repetition resulted in increased tolerance. In the first series of experiments the injections were usually made only after recovery from the effects of previous doses. A variety of different doses was tried, given at intervals which varied from 45 minutes to 100 hours and repeated 2 to 4 times. In such experiments on 34 cats, there was no clear indication of a change in the sensitivity to physostigmine with repeated injections. Of 22 animals which received 1 mg. after treatment with smaller amounts, 18, or 82 per cent, died. This is a slight but probably insignificant increase in survival rate from the 1 mg. dose of physostigmine which in the control series was an LD 94. Survival time, severity of symptoms, and rate of recovery were indistinguishable in the two groups.

In experiments to be described later it was found that the prophylactic action

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² This study is part of a cooperative investigation planned and carried out by McKeen Cattell, Harry Gold, and other workers in the Department of Pharmacology. The paper is published under the name of the chief collaborator.

³ In this paper all doses are expressed in terms of body weight in kilograms.

of physostigmine against DFP poisoning was not improved by pre-treatment with more than one dose of physostigmine and that the best protection was obtained when the DFP was given within a few minutes after the physostigmine. Therefore in a second series of experiments the test dose of 1 mg. of physostigmine was given within 1 to 7 minutes after a small initial test dose (0.05 and 0.1 mg.). The results for 25 animals are given in table 2. Five of the group of 20 cats receiving a conditioning dose of 0.1 or 0.05 mg. survived, giving a mortality of 75 per cent as against 94 per cent for the controls. The total physostigmine (1.1 and 1.05 mg.) received and the survival rate were both slightly greater as compared to the controls receiving 1 mg. in a single dose. It may be questioned

TABLE 1
Toxicity of physostigmine by intravenous injection

NO. OF CATS	DOSE	MORTALITY	TIME TO DEATH	
			Average	Range
	mg./kg.		min.	
16	1.0	15/16	22*	2-77*
24	0.5	5/24	51*	10-93*
16	0.4	0/16		
13	0.25	0/13		

* Excluding 1 cat which lived 6 hours but died overnight.

TABLE 2
Toxicity of intravenous physostigmine preceded by a small dose of the same drug

NO. OF CATS	PHYSOSTIGMINE		INTERVAL BETWEEN DOSES	MORTALITY
	Conditioning dose	Test dose		
	mg./kg.	mg./kg.	min.	
16		1		15/16 = 94%
6	0.1	1	3-7	5/6 = 83%
14	0.05	1	1-6	10/14 = 71%
4	0.01	1	4-13	4/4 = 100%
1	0.001	1	4	1/1

whether this represents a significant increase in tolerance; at best it is but slight in comparison with the high degree of protection afforded against DFP by pre-treatment with physostigmine.

Effect of repeated doses of DFP. Data obtained from cats receiving repeated doses of DFP by vein at various intervals are assembled in table 3. The dose employed (1 mg.) was approximately 0.6 of an LD 50 and in 12 cats resulted in no deaths from a single injection. A repetition of the dose within a few days resulted in a number of deaths, and a third dose given to survivors killed 3 out of 4 animals. Since DFP is rapidly destroyed in the body (1) but the reduction in cholinesterase activity of the blood plasma and tissues is long lasting (2), it may

be presumed that the latter effect is responsible for the increased sensitivity of cats to repeated doses of DFP.

Toxicity of DFP after physostigmine. In studying the relationship between physostigmine and DFP, a variety of dosage regimens was explored. In the earlier experiments, from 3 to 4 doses of physostigmine (0.5 or 1 mg.) were given on the assumption that by repeating the dosage a higher resistance might be obtained. However, it soon appeared that the results were independent of the number of doses of physostigmine given, and in later experiments the DFP was given at various intervals following a single dose.

Of 11 cats receiving their first dose of DFP by vein (approximately 3 times an LD 50) from 1½ to 73 hours after the last dose of physostigmine, 7 survived and those which died in many instances lived longer than the controls. Three cats were given the dose of DFP within a few minutes of the physostigmine (0.25 mg.) and all these survived, a result suggesting the superiority of shorter intervals.

TABLE 3
Repeated doses of 1 mg./kg. di-isopropyl fluorophosphate

DOSES	INTERVAL	NO. OF CATS	PER CENT MORTALITY
First		12	0
Second	1-2 hrs.	4	0
	1-3 days	6	33
	7 days	2	0
Third	1 day	4	75
	5-7 days	6	0
Fourth	8 days	2	0

The data from further series of experiments on the influence of pre-treatment with physostigmine on the toxicity of DFP are presented in table 4. The LD 50 dose of this specimen of DFP determined in 78 cats was 1.7 mg. In the analysis of the extent of protection, three factors are important, viz., the dose of physostigmine, the dose of DFP, and the interval between injections. With 10 mg. of DFP, about 6 times the LD 50 dose, only an occasional animal survived. With 7 mg., excellent protection was evidenced provided the period intervening since the injection of physostigmine was short. In the group in which the interval was from 1½ to 2½ hours after physostigmine (0.05 mg.) all of the seven animals died, although they lived much longer than untreated controls which died in from 4 to 5 minutes. For all doses of DFP the best results were obtained when the interval between injections was only a few minutes; however, under these conditions there was a factor of added toxicity and the best results were obtained with the smaller doses of physostigmine (0.05 to 0.1 mg.). When the dose of physostigmine was reduced to 0.01 mg., there was no evidence of

protection, and because of the added toxicity at short intervals, the optimum dose appears to be about 0.05 to 0.1 mg. Animals which survived 3 to 6 LD 50 doses of DFP usually exhibited rather severe symptoms, but recovered more rapidly than unprotected animals receiving smaller doses. The following day most of them appeared normal, but later approximately half developed chronic symptoms (weakness, ataxia, pilomotor stimulation) characteristic of delayed DFP poisoning.

TABLE 4
Effect of physostigmine on the toxicity of di-isopropyl fluorophosphate

NO. OF CATS	PHYSO-STIGMINE mg./kg.	DFP mg./kg.	INTERVAL BETWEEN INJECTIONS	MOR- TALITY	TIME TO DEATH	
					Average	Range
4	0.1	10	2½-3 min.	3/4		2½ & 90 min.*
4	0.25	10	1½-5½ min.	3/4		Immediate, 10 & 42 min.
3	0.25	10	2½-2½ hrs.	3/3	16 min.	8-25 min.
2	0.25	10	95-101 hrs.	2/2	5½ min.	3-8 min.
5	0.01	7	2-10 min.	5/5	8 min.	2-19 min.
6	0.05	7	2-14 min.	2/6	20 min.	10-30 min.
7	0.05	7	1½-2½ hrs.	7/7	37 min.	4, 150 min.
8	0.1	7	2½-8 min.	1/8	102 min.	
6	0.1	7	2-2½ hrs.	4/6	120+ min.†	99-142 min.†
6	0.1	7	5-6½ hrs.	6/6	7 min.	2-20 min.
10	0.25	7	3½-8½ min.	4/10	2½ min.	1½-3 min.
7	0.25	7	3½-4 hrs.	5/7		5, 101 & 195 min.‡
3	0.4	7	2½-4 min.	2/3	95 min.	72-118 min.
3	0.4	7	5 hrs.	1/3	22+ hrs.§	
7	0.4	7	8 hrs.	7/7	10 min.	4-33 min.
3	0.1	5	4-5½ min.	0/3		

* Excluding one cat which lived at least one hour but died overnight.

† Excluding two cats which lived at least 1½ and 3 hours but died overnight.

‡ Excluding two cats which lived at least 1 and 4 hours but died overnight.

§ Died during weekend.

Toxicity of DFP after atropine plus physostigmine. By giving a small dose of atropine before physostigmine, it is possible to prevent the peripheral autonomic effects of the latter drug and this enables the injection of larger doses. This has been done in a few experiments to explore further the degree of protection obtainable against DFP poisoning. Small doses of atropine, such as those employed in the present experiments, afford only a moderate degree of protection in cats (3). The larger doses of physostigmine following atropine resulted in a very high degree of protection against DFP as shown in table 5. With one ex-

ception, in these experiments the DFP was given after an interval of from 3 to 4½ hours when all symptoms from the physostigmine had subsided. Animals were protected against a dose as high as 50 mg. of DFP (30 times an LD 50), and in these animals symptoms were comparatively mild or entirely absent. In cat no. 273 (table 5) in which the injection of DFP (7 mg.) was delayed for 23 hours after the physostigmine, there was no protection.

TABLE 5
Atropine-physostigmine protection against DFP

CAT NO.	ATRO- PINE	PHYSO- STIG- MINE	INTERVAL AFTER PHY- SOSTIGMINE	DFP	RESULTS
	mg./kg.	mg./kg.	hours	mg./kg.	
272	0.1	1	4½	7	Appears normal, no symptoms. Normal and lively 19 hours later.
274	0.1	1	3½	7	Only effect is very slight restlessness, lasting 4 min. Normal and lively 19 hours later.
273	0.1	1	23	7	Dies in 4 minutes.
345	0.2	2	3	20	2 min.: lying down, sl. dyspnea, sl. gen. fibr. 8 min.: defecation, sitting, sl. tense, very few fibr. 14 min.: sitting, entirely normal. 52 min.: appears normal, but unable to stand. 19½ hrs.: entirely normal and lively.
346	0.2	2	3	28.7	5 min.: Restless, gen. fibr. 8 min.: Quiet, sl. irreg. resp., occasional fibr., 22 min.: normal except a few fibr., stands but weak. 49 hrs.: normal except sl. ataxia. 69 hrs.: sl. ataxia. 114 hrs.: found dead.
350	0.3	2	3½	50	1 min.: fibr., marked dyspnea, hyperexcitability. 3 min.: no fibr., less dysp., jerks with atypical movements. 6 min.: lying down, no fibr., slow, labored resp. 20 min.: lies entirely quiet, occasional fibr., resp. normal. 52 min.: lies on side unable to stand. 17 hrs.: alert, active, marked ataxia, 44 hrs.: same. 4 days: same but only sl. ataxia. 6 days: same, sl. to moderate ataxia.
349	0.3	2	3½	50	2 min.: sits quiet, paws folded on chest. 20 min.: never any symptoms, now walks normally. 55 min.: walks normally except sl. leg tremors, cries repeatedly. 24 hrs.: alert but walks with difficulty. 4 days: much improved, walks with sl. ataxia. 6 days: walks slowly with sl. ataxia.

In view of the limited protection afforded by small doses of atropine alone, it is probable that the very high degree of protection obtained in these experiments was due to the larger doses of physostigmine employed.

Toxicity of physostigmine after DFP. When physostigmine was administered to cats which had been given DFP previously, instead of protection as was the case when given in the reverse order, it was more toxic than in normal cats. The physostigmine alone had an LD 50 of 0.66 mg.; all survived 0.4 mg., while

21 per cent died after 0.5 mg. in an average period of 51 minutes. The greater susceptibility of the DFP-treated animals is clearly shown in the data summarized in table 6. It may be noted that one-half of the LD 21 (0.25 mg.) of physostigmine became an LD 70 after DFP. The dose of 0.25 mg. of physostigmine which was survived by all 13 control cats proved fatal in 89 per cent of the 9 cats receiving 3.5 mg. of DFP. In the DFP-poisoned animals, the physostigmine proved fatal in an average of about 10 minutes or one-fifth of the time in the control cats receiving 0.5 mg. The physostigmine was administered one or more days after the DFP when the animals had fully recovered from the acute effects and in most instances also from any chronic symptoms. In one cat there were some persisting symptoms of DFP poisoning 5 months later and at that time the injection of 0.25 mg. caused death, showing a long-lasting susceptibility to physostigmine. In the case of the larger doses of DFP, atropine with magnesium sulfate was used to protect the animals against the fatal action of DFP.

TABLE 6
Toxicity of physostigmine following di-isopropyl fluorophosphate

NO. OF CATS	PROTECTIVE AGENT	DOSE OF FLUOROPHOSPHATE	INTERVAL	DOSE OF PHYSOSTIGMINE	PER CENT MORTALITY
		mg./kg.		mg./kg.	
13	None	None		0.25	0
4	None	1.5	6 days	0.25	50
7	None	1	2-31 min.	0.25	71
3	None	1	2 days	0.25	0
3	Atropine	3	1-2 days	0.25	100
9	Atropine + magnesium	3.5*	3-11 days	0.25	89
9	Physostigmine	5*	1-11 days	0.25	22
11	Physostigmine	7	1-7 days	0.25	9

* This sample of DFP was sub-standard. The figure represents milligrams of the standard material having an activity equal to that of the dose given.

These animals showed the same increased susceptibility to physostigmine as the animals which survived the smaller doses of DFP without protective agents.

In the case of the animals protected by physostigmine, DFP resulted in a much smaller increase in susceptibility to the subsequent dose of physostigmine. The dose of 0.25 mg. of physostigmine, which was an LD 89 in the animals protected with atropine plus magnesium, was only an LD 15 in those protected with physostigmine.

The increased susceptibility of cats to the action of physostigmine following DFP is in all probability due to the destruction of cholinesterase by the latter drug. A small further reduction of the esterase activity by physostigmine might be expected to cause symptoms.

The failure of the animals treated with physostigmine to show the same degree of sensitivity to a further dose of physostigmine given after DFP is presumably to be ascribed to the protection of the cholinesterase by the first dose of physostigmine, evidence for which is presented in the next section.

Protection of cholinesterase by physostigmine. The probable mechanism through which physostigmine protects against toxic action of DFP has been revealed in an investigation carried out by Koelle, the results of which are to be published in a following paper (4). It was found that physostigmine completely protected the serum cholinesterase in vitro from the irreversible inactivation produced by DFP, as demonstrated by the fact that full activity was regained by the physostigmine and DFP treated serum following dialysis. We have since carried out experiments which demonstrate the same phenomenon in intact cats.

In the present experiments the animals were given an intravenous dose of physostigmine which was followed within 15 to 30 minutes by DFP, also given

TABLE 7
Protection of serum cholinesterase by physostigmine

CAT NO.	ATROPINE	PHYSOSTIG-MINE	ESTERASE ACTIVITY AFTER PHYSOSTIGMINE % OF CONTROL	DFP	ESTERASE ACTIVITY TIME AFTER DFP		
					20 min. \pm	4-6 hrs.	24 hrs.
	mg./kg.	mg./kg.		mg./kg.			
394				0.1			32
390a				0.1	14	16	33
390b				0.1			40
420	1			0.1	18	26	42
423	1			0.1	24	29	29
428				0.1	16	23	38
392				0.2	22	24	24
397				0.7	9	7	20
389a		0.1	72	0.1	48	77	76
389b		0.1	80	0.1	49	73	68
393		0.1	68	0.1	37	68	58
421	1	2.0	52	0.1	47	55	68
422	1	2.0	56	0.1	42	52	71
427		0.1		0.1	39	51	47
391		0.1	73	0.2	34	28	30
396		0.1	73	0.7	18	11	15
300		0.1	67	3.0	7		16
424		0.1	78	3.0		6	27

by vein. In two of the animals atropine was given to permit the use of a larger dose of physostigmine. Blood samples were obtained just before the physostigmine was injected (control), just before the DFP, and at various intervals thereafter. Serum cholinesterase was determined by the manometric technic under uniform conditions of dilution, etc., and the results expressed as percentages of the control value.

The results are presented in table 7. The figures in the upper half of the table are for cats receiving only DFP (sometimes with atropine), while those in the lower portion represent animals protected with physostigmine. In comparing the two groups it will be noted that in the absence of physostigmine the

cholinesterase activity fell to a low value following the DFP and there was no appreciable recovery during the following 4 to 6 hour period. In contrast, the animals protected with physostigmine, which reduced the cholinesterase activity by about 25%, not only had a much higher serum cholinesterase activity after the DFP, but in the subsequent 4 to 6 hour period regained considerable activity. Thus several hours after the 0.1 mg. dose of DFP, the serum activity of the physostigmine-protected animals averaged about 63% of its original value, compared to about 25% in the untreated group.

In the case of the animals receiving larger doses of DFP, the serum cholinesterase was not appreciably protected by physostigmine. However, without further experiments this cannot be taken as evidence against the operation of a similar protective mechanism of physostigmine in saving animals from large doses of DFP. There is much evidence to indicate that the plasma cholinesterase bears little relation to the toxic effects of DFP (see Koelle and Gilman, 5). The reduction in tissue esterase, particularly that of the brain, appears to be the determining factor in toxicity, and in general brain cholinesterase is more resistant to DFP than that of the plasma. It is therefore not improbable that the doses of physostigmine employed may be sufficient to prevent a critical depression of the brain esterase, and thus allow recovery from otherwise fatal doses of DFP.

SUMMARY

1. A small dose of physostigmine given to cats protects against the fatal action of a subsequent dose of DFP (di-isopropyl fluorophosphate).
2. Repeated injections of physostigmine do not significantly increase tolerance.
3. DFP results in a long lasting increase in the susceptibility of cats to the lethal action of physostigmine. This result of DFP is much less pronounced in cats protected by physostigmine.
4. The cholinesterase activity of the serum from animals pre-treated with physostigmine shows a smaller reduction than serum from animals receiving DFP alone, and recovery is more rapid.

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PHARMACOLOGICAL AND CHEMOTHERAPEUTIC PROPERTIES OF 3,4-DIMETHYL-5- SULFANILAMIDO-ISOXAZOLE

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Backer and de Jonge (1) first described the synthesis of 3-methyl-5-sulfanilamido-isoxazole and mentioned that this compound though its anticomoccal activity was slight, appeared promising. Andersen, Faith, Marson, Winnek and Roblin Jr. (2) described the same compound which exerted a slight anticomoccal activity *in vivo*, but was found to be of appreciable activity *in vitro* towards *E. coli*. Independently of these investigations an identical product had been prepared by M. Hoffer and H. M. Wuest in the Roche Chemical Research Laboratories. The biological evaluation of this compound confirmed the findings of Backer and de Jonge as well as those of Anderson and his co-workers as far as animal experiments with hemolytic streptococci were concerned. The observation, however, that pneumococci showed a somewhat better response to 3-methyl-5-sulfanilamido-isoxazole than streptococci and the comparatively high water solubility of this compound prompted an extended chemical and biological study of the group of N¹-sulfanilamido-isoxazole derivatives. From the investigation of numerous members of this series undertaken during recent years it was observed, that blocking of the 4-position in the isoxazole ring by an alkyl-group increased the antibacterial properties, and one compound, 3,4-dimethyl-5-sulfanilamido-isoxazole (Nu-445) seemed to possess not only satisfactory therapeutic activity in experimental infections with gram-positive and gram-negative organisms, but particularly favorable properties of solubility within the physiologically important range of pH 6.0-7.5. It could, therefore, be expected that crystallization in the kidneys and the subsequent pathological conditions due to the low solubility of other sulfonamides could be avoided if such a compound of exceptionally high solubility at neutral reaction was used.

For that reason an experimental study of the pharmacological and chemotherapeutic properties of 3,4-dimethyl-5-sulfanilamido-isoxazole was carried out, the results of which are presented in this paper.

MATERIAL AND METHODS. The majority of the experiments were done in albino mice of 16-22 g. of weight. For special toxicological tests young white rats of 80-90 g. and rabbits of 3-4 kg. weight were used.

Toxicological investigation: The oral, subcutaneous, intravenous, and intra-abdominal routes of administration were used. In experiments with chronic oral or parenteral administration of the compound necropsy of all animals was performed and supplemented by histological examination of liver, kidneys and stomachs. During the course of treatment of rabbits the blood was examined at regular intervals, and hemoglobin determination as

well as erythrocyte, leucocyte count and differential count of the white cells were carried out.

The concentration of the compound in the blood and urine was determined according to Bratton and Marshall (3).

Chemotherapeutic experiments were carried out with the following strains:

β-Hemolytic streptococci (Group A)

Strept. 4 type 3

Strept. C 203 type 3¹

Strept. 646 type 3¹

Strept. Griffith 1 type 1² (Marked G₁ in the tables)

Strept. Griffith 2 type 2² (Marked G₂ in the tables)

Staphylococcus aureus

Staph. 6340²

Staph. B 8

Pneumococci

Pn. 6301 type I²

Pn. 6302 type II²

Pn. 6303 type III²

Meningococcus Group I (1 strain)²

Klebsiella Group A² (1 strain)

C. diphtheriae #9060 type gravis²

Organisms of the Coli-typhoid group

E. coli J.

*S. schottmuelleri*²

S. typhi-murium.²

In all instances intra-abdominal infection of mice with 100-1000 MLD of the various organisms was used in our experiments. Details of the techniques of infection and treatment as used in this laboratory were described earlier for streptococcal (4), pneumococcal (5), staphylococcal (6) infections and for infections with *C. diphtheriae* (7). Similar procedures were adopted for infecting mice with the other organisms; Meningococci and the members of the coli-salmonella group were administered in 5% mucin suspension.

The general schedule of treatment consisted in 2 treatments on the first day, one of them shortly after the infection; 2 more treatments were given on the second day and 1 treatment on each the 3rd and 4th day. In many instances the activity of one single treatment was also determined. Single treatment was the rule in the meningococcal experiments; here the drug was administered 2 hours before the infection.

Other sulfonamides, particularly sulfathiazole and sulfadiazine were run as comparison in many experiments. Groups of 5-10 mice were used for every drug and dose.

All mice succumbing to the infection were autopsied and cultured. Surviving animals were under observation for periods of 3 to 4 weeks.

Further details will be found in the experimental part.

3,4-dimethyl-5-sulfanilamido-isoxazole (Nu-445) was studied as free sulfonamide, generally dissolved in neutral buffer solution or (for oral treatment) in 5% gum acacia. Neutral salts of the compound such as the salts of sodium, lithium, calcium, ethanalamine, di-ethanalamine and ethylene diamine were also investigated. As there were no essential differences in toxicity or activity of the different salts only the properties of the Na- and Li-salts will be described besides those of the free sulfonamide.

¹ This strain was obtained by the kindness of Dr. C. A. Lawrence, The Winthrop Chemical Co. Rensselaer, N. Y.

² Received from the American Type Culture Collection.

³ Kindly given to us by Dr. E. A. Kabat, Coll. of Physicians and Surgeons Columbia Univ. New York.

EXPERIMENTAL. Pharmacological results. (a) *Acute toxicity.* The LD50-doses evaluated in mice and rats by different routes of administration are given in table 1. The doses for parenteral application are higher than those of other N¹-substituted sulfonamides which are generally found in the range 1.0–1.5 g./kg. This is probably due to the fact that the isoxazole derivative and its salts are soluble in high concentrations at neutral or only slightly alkaline reaction (a 11% aqueous solution of the sodium salt has pH 7.2). For the same reason the oral LD50 doses were found identical for the salts and the free sulfonamide.

(b) *Chronic toxicity. Rats.* Twenty four young rats of 82–93 g. of weight were put on a diet of powdered Purina dog chow containing 2% of Nu-445. The animals were weighed weekly and a record was kept of the food consumption of the individual rats. The animals were killed at different intervals, autopsied and sections of liver, kidney and stomach were taken. The experiments covered a period of more than half a year. The last group of 4 animals sacrificed after 182 days received a total dosage as high as 245 g./kg. (average daily intake 1.35

TABLE 1
LD50 doses of 3,4-dimethyl-5 sulfanilamido-isoxazole
(g./kg. calculated as free sulfonamide)

COMPOUND	SPECIES	INTRAVENOUS	INTRAPERITONEAL	SUBCUTANEOUS	ORAL
Nu-445	mouse				10
Sodium salt	mouse	2.3		2.8*	10
	rat		3.2		10
Lithium salt	mouse	2.5		5.0	10

* 100% tolerated dose.

g./kg.). No macroscopic or microscopic changes attributable to the drug could be found in any of the animals kept on the drug diet, nor was an inhibition of growth noticed in these animals (table 2). Of particular interest was the observation that there were no calculi in the kidneys, nor evidence of their previous existence.

Rabbits. Daily subcutaneous injection of 0.5–1.0 g./kg. of the sodium salt for a period of about 4 weeks produced no significant weight changes in 5 rabbits weighing 3.5–4.0 kgs. The number and form of blood corpuscles, differential count and hemoglobin were examined during the whole duration of the treatment and afterwards. There were no significant changes in the blood pictures of these rabbits followed up to the 19th day after the last injection (table 3).

The red and the white count were always within normal range. The differential count of the white cells hardly changed at all during the extensive treatment. The average count of granulocytes before onset of the treatment was 27%, and 65% lymphocytes were found. After 4 weeks treatment the corres-

TABLE 2
Average weights of rats on preparation Nu-445

WEEKS	NORMAL DIET				DIET WITH 2% NU-445				
	Group								
	1	2	3	4	5	6	7	8	9
	Rat numbers								
	1-2	3-4	5-6	7-10	11-14	15-18	19-22	23-26	27-30
0	91	82	87	93	93	80	93	92	86
1	99	99	106	117	110	98	100	112	108
2	111	110	119		125	115	113	129	126
3	128	133	128			135	137	151	145
4	145	159	149			144	155	165	159
5		155	153				168	176	173
6		168	162				183	192	181
7			167					200	193
8			168					208	206
9			171					207	218
10									226
11									229
12									237
26									275
Av. daily food per rat—g.	10.9	13.7	12.2	11.4	12.0	11.3	11.1	12.8	14.7
Av. daily drug per kg. —g.				2.19	2.19	1.97	1.57	1.58	1.35
Av. total drug per kg.—g..				15.3	30.7	55.6	66.1	85.1	245.7

TABLE 3
Blood counts of rabbits treated with Nu-445 Na salt

Total dose 11-22 g./kg. s.c.

TREATMENT PERIOD	DURATION OF TREATMENT	NUMBER OF TESTS	NO. OF RABBITS	RBC $\times 10^6$	Hgb	WBC $\times 10^4$
	weeks				grams	
Before...		4	5	7.24	13.1	8.7
During.....	1-2	5	5	6.16	11.3	8.8
During...	3-4	4	5	5.98	10.3	8.4
After	1-2	3*	4	6.01	10.2	8.9

* 6, 9 and 16 days after last injection.

ponding figures were 28% granulocytes and 68% lymphocytes, 19 days after the termination of the treatment the figures had not changed; 28% granulocytes and 70% lymphocytes were counted.

The possible effect of the drug on the kidneys was investigated in another group of 6 rabbits, by daily oral administration of 0.794 g./kg. of the sodium salt. Hemoglobin, urine output and pH, body weight, and blood and urine level (free and total) of the drug were observed. The animals were sacrificed after 10 days and post mortem examinations made. The values obtained for 'free' sulfonamide concentration in the urine varied between 105 and 797 mg. % (average 352 mg. % of 20 determinations), and for 'total' sulfonamide, between 212 and 1190 mg. % (average 520 mg. %). In spite of the high amount of sulfonamide excreted through the urine, no crystalline deposits in the kidney or other gross or microscopical pathological changes were noticed. The pH of the urine remained on a constant level of pH 8.4. It should be mentioned that under the same experimental conditions, crystallization in the kidneys of rabbits followed the

TABLE 4

Blood level of Nu-445 in mg% after oral and subcutaneous treatment of mice

COMPOUND	ROUTE	DOSE	HOURS				
		g./kg.	$\frac{1}{2}$	1 $\frac{1}{2}$	2 $\frac{1}{2}$	3 $\frac{1}{2}$	5
Nu-445	oral	0.025	3.5	4	1.5	0.8	0
		0.050	8.8	4	3.8	2.6	1.4
		0.125	12	11.5		4.5	2.5
	subcut.	0.025	7.5	3.7	1.5	0.5	0
		0.050	10.5	5.0	3.5	3.0	1
		0.125	12	9	8.5	3.7	2
Li-salt	oral	0.0125	4	2.5	trace		
		0.025	4.5	3	2.5		
		0.050	7	5	3	0.5	trace
		0.125	11	5	4	4	3.5
		0.250	15	13	6	6	2
	subcut.	0.0125	4.5	3.5	2.5		
		0.025	8	2	2		
		0.050	10	7	4	0.5	trace
		0.250	14	8.5	3.5	3	1.5

oral administration of most sulfonamides even if given in smaller doses (0.5 g./kg.) with consequent oliguria and acid reaction of the urines. The pathological changes of the kidneys influenced in these cases the blood concentrations too; they tended to become extremely high, while in animals treated with Nu-445 the blood level remained constant (see section 'absorption,' below).

(c) *Absorption and elimination. Mice.* The blood levels observed after oral and subcutaneous treatment of mice with 3,4-dimethyl-5-sulfanilamido-isoxazole and its Li-salt are given in table 4. The blood concentrations seemed to be of about the same order as those previously reported for other N¹-substituted sulfonamides (6). The intravenous administration of 0.050 g./kg. of Nu-445 gave a blood concentration of 8 mg. % half an hour after administration, which dropped to 4.5-5 mg. % after 1 $\frac{1}{2}$ -2 $\frac{1}{2}$ hours; in the determination made after 3 $\frac{1}{2}$

hours 2.2 mg. % were still found in the blood. No diazotizable material could be detected 5 hours after intravenous injection.

Rats. Young animals kept on a diet containing 2% Nu-445 have shown a constant blood level of 12 to 18 mg. %, as determined on samples taken from rats sacrificed after they were kept on the diet for 1, 2, 4 and 6 weeks.

Rabbits. Daily oral administration of 0.794 g./kg. of the sodium salt gave blood concentrations which rose from 10 mg. % to 20 mg. % on the 8th day. The average total drug excreted through the urine during the first day was 344 mg./kg. and during the 4 to 10 day period 488 mg./kg. per day. Thus, 43-61% of the drug administered orally was eliminated through the kidneys and the larger part of it (68%) in unacetylated form.

These results which were obtained with the Bratton-Marshall method were confirmed by the chemical isolation of the sulfonamide and its acetyl-derivative from the urine (Dr. M. Hoffer). The ratio of free to acetylated dimethyl-sulfanilamido-soxazole was 2:1.

One rabbit which received daily 1.0 g./kg. of the sodium salt, examined 4 hours after the third subcutaneous injection, showed a spinal fluid concentration of 3 mg. % (occipital puncture) with a blood level of 10 mg. %

(d) *Local irritation.* Subcutaneous injections of 10% and 20% sodium salt were made in the dorsal skin of the rabbit up to volumes of 24 cc. and intradermal injections of 0.02 cc. in the skin of the ears. In no cases was there irritation that could be considered significant.

In order to decide whether the drug might be used in the peritoneal cavity, laparotomy was performed on 4 rabbits under ether anesthesia and the powdered drug placed in the peritoneal cavity. Two animals treated with 0.5 and 0.2 gm./kgm. of the powdered lithium salt, sacrificed after 2 and 5 days respectively showed no gross pathological changes. The peritoneum was smooth and glossy and did not indicate inflammation. In one rabbit, however, a few small hemorrhages were visible, apparently in the subserous layer of the duodenum and the descendent colon. The histological examination showed that the small hemorrhages were in the deeper layers of the intestinal wall and probably due to accidental injury. The absorption of the crystalline powder from the peritoneal cavity took place very rapidly. High blood concentrations (up to 40 mg. %) were reached 1 hour after the peritoneal application. It was interesting that washings of the peritoneal cavity 42 hours after administration still contained a diazotizable substance, although none could be detected in the blood stream after 17 hours. Two rabbits treated with 0.1 and 0.2 gm./kgm. of the free sulfonamide showed no adhesions or pathological changes in the peritoneal cavity when sacrificed 2½ months after application of the drug.

Chemotherapeutic results. (a) *bacteriostatic activity in vitro.* The technique used in these experiments was the dilution method with serial dilutions in different media. The pathogenic cocci were tested in papain digest broth (8), the organisms of the coli-typhoid group as well as *B. proteus* were tested in addition in the synthetic medium described by Spink and Vivino (9).

As table 5 indicates, Nu-445 and its salts have a moderate activity in vitro

against gram positive organisms, but they possess a pronounced bacteriostatic effect against gram negative bacteria. The higher activity against *E. typhi* in synthetic medium than in broth is probably due to the presence of inhibiting substances in the latter medium. The bacteriostatic effect against *E. coli* could be almost completely inhibited by p-aminobenzoic acid: in the presence of 5 mg.% p-aminobenzoic acid in the medium approximately 500 times the concentration was required for bacteriostasis. It was furthermore found that the bacteriostatic effect against *E. coli*, *Klebsiella* and *B. proteus vulg.* was greatly

TABLE 5
In vitro activity of Nu-445 lithium salt

ORGANISM	BACTERIOSTATIC CONCENTRATION IN	
	Broth	Synthetic medium
Strept. hem.....	1- 200	
Staph. aureus.....	1- 100	
Pneumococcus I.....	1- 800	
Pneumococcus II.....	1- 1600	
Pneumococcus III.....	1- 800	
<i>E. coli</i>	1-64,000	1- 64,000
<i>E. typhi</i>	1-20,000	1-320,000
<i>B. proteus vulgaris</i>		1- 12,000
<i>Klebsiella A</i>		1- 51,200

TABLE 6

50% active doses of Nu-445 and its lithium salt in the streptococcus hemolyticus infections of mice

STREPT. STRAIN	GROUP A TYPE NO.	50% ACTIVE DOSE G.KG.*		
		Nu-445 oral	Li-salt oral	Li-salt s.c.
4	3	0.05	0.0125	0.025
646	3	0.125	0.05	0.0125
C203	3	0.125	0.125	0.05
GI	1	0.05	0.0125	>0.025
GII	2	0.025		

* Single dose repeated six times.

influenced by the pH of the medium. The optimum zone was found between pH 6.2 and 7.0; at pH 8.4 the drop of activity was very marked.

(b) *Antistreptococcal activity in vivo.* In table 6 the 50% active doses of Nu-445 and its Li-salt are given. The value are based on experiments with more than 1500 mice, and it can be seen that the compound exerts a marked activity against all 3 types of Group A strains tested. It is noteworthy that the free sulfonamide was somewhat less effective than the lithium salt. This difference of activity cannot be satisfactorily explained, particularly not on the basis of the blood level. The sensitivity of the different strains to treatment was different, in agreement with our experience on other sulfonamide derivatives.

(c) *Antipneumococcal activity in vivo.* Experiments conducted in mice infected with 1000 MLD of pneumococci showed activity against all three types of strains (table 7). In the type I and III infections the average active dose was 1.5-2.5 g./kg.; lower doses gave a significant delay of death. In the type II infection the activity was more pronounced with the free compound than with the lithium salt which gave delay of death up to 5 days (compared to 26 hours in the control group) but only few final survivors were observed. The higher

TABLE 7

Antipneumococcal activity of Nu-445 and its Li-salt by oral administration

PNEUMO.	TYPE	DRUG	DOSE	NO. OF MICE	FINAL SURVIVORS
			g./kg.		%
6301	I	Nu-445	2.0-2.5	30	64
			1.5	10	70
			1.0	40	17
			0.5	20	0
		Li-salt	2.5	10	100
			1.5	40	50
			0.5	30	26
		Controls		65	1.8
6302	II	Nu-445	2.0-2.5	25	50
			1.5	10	70
			1.0	20	0
			0.5	10	0
		Li-salt	2.5	17	18
			1.5	10	10
			0.5	10	0
		Controls		25	0
6303	III	Nu-445	2.0-2.5	18	50
			1.0	20	30
			0.5	10	0
		Li-salt	2.5	8	50
			1.5	20	20
			0.5	10	0
		Controls		25	0

resistance of the type II infection to treatment was in agreement with our experience on other sulfonamides.

(d) *Antistaphylococcal activity in vivo.* The minimal (50%) active dose by single treatment was 0.125 g./kg. for the free compound and 0.025 g./kg. for the free compound and 0.025 g./kg. or less for the lithium salt in the infection with 100 MLD Staph. aureus 6340. Similar results were obtained with the lithium salt in the infection with the strain B8.

(e) *Antidiphtherial activity.* In the infection with 10-100 MLD of *C. diphtheriae* (7) a single oral treatment with 0.05 g./kg. protected 60% of the mice.

(f) *Antimeningococcal activity in vivo.* In these experiments (table 8) a single oral treatment was given 2 hours before the intra-abdominal infection with 100

MLD. The suspension of meningococci was made up from 6 hours cultures on blood agar slants and adjusted to a standard turbidity of 10% light transmission determined in a Lumetron photo electrometer. Proper dilutions, generally

TABLE 8

Activity of a single oral treatment with Nu-445 (Li-salt) in meningococcal infection of mice
Infection: 1 cc. 10^{-4} dilution in mucin of a 6 hours' growth on blood agar slants.

NU-445 g./kg.	NUMBER OF		SURVIVORS %
	Mice	Survivors	
0.050	30	29	96
0.0166	20	17	85
0.010	40	27	68
0.005	40	19	47
Controls	70	3	4.3

TABLE 9

Activity of Nu-445 lithium salt in infections with gram negative organisms of the coli-typhoid groups (including *Klebsiella*)

INFECTION	DOSE g./kg.	NUMBER OF TREATMENTS	NO. OF MICE	SURVIVORS %
<i>S. schottmuelleri</i>	1.0	1	5	100
	0.5	1	5	60
	0.25	1	5	60
	0.125	1	5	60
	0.06	1	5	40
	Controls		59	1.7
<i>Klebsiella A</i>	1.25	5	5	100
	2.5	2	5	0
	0.5	6	5	0
	Controls		25	8
<i>E. coli</i>	1.25	6	5	100
	0.5	6	5	80
	1.25	1	10	50
	0.5	1	15	70
	0.25-0.1	1	10	40
	Controls		15	7

10^{-4} to 10^{-5} were prepared with 5% mucin, and the animals injected with 1.0 cc. of these suspensions. Untreated controls died within 30 hours.

(g) Activity in *Klebsiella* infections and in infections with *E. coli* and *S. schottmuelleri*. As might be seen from table 9, the lithium salt of Nu-445 exerted a definite action in experimental infections with organisms of the coli-typhoid group including *Klebsiella* group A. The minimal (50%) active doses in *E.*

coli and *Salmonella* infections were about 0.1 mg./kg., while the highly virulent strain of *Klebsiella* required total doses between 5 and 7 g./kg.

The anti-*coli* effect of the drug was also very definitely demonstrated in the rats kept on the 2% medicated diet. During the experiments on chronic toxicity the intestinal *coli*-count of these animals dropped considerably. Some animals showed only 0-10 colonies on the counting plates. The highest count was 188 *coli* colonies, while the untreated controls showed development of innumerable colonies.

Another *Salmonella* strain *S. typhi-murium* was less sensitive to treatment with Nu-445. In oral infections with this strain no final survivors were observed, even though the death of the animals was delayed up to 12-20 days. None of the known sulfonamides was found to be active in the infection with this strain of *Salmonella*.

TABLE 10

Comparison of the average minimal (50%) active doses of sulfathiazole, sulfadiazine and 3,4-di-methyl-5-sulfanilamido-isoxazole (Nu-445)

Total doses for oral treatment in g./kg.

ORGANISMS	SULFATHIAZOLE	SULFADIAZINE	NU-445
Hem. strept.	0.79	0.15	0.3
Pneumococci I	>15 0	6 0	8.0
Pneumococci II		15 0	8.0
Pneumococci III	>15 0	15 0	15.0
Staph. aureus	0.125	0.025	0.025
C. diphtheriae	0.125	0 05	0.05
Meningococci	0 2	0 003	0.005
<i>S. schottmuelleri</i>		0 025	0.06
<i>E. coli</i>		0.1	0.15
<i>Klebsiella A</i>		0 3	7.5

DISCUSSION. It was demonstrated in the experimental part that 3,4-di-methyl-5-sulfanilamido-isoxazole (Nu-445) had a low toxicity by oral and parenteral administration due, probably, to its ready solubility in water at neutral reaction. There was no evidence in the experiments in rats and rabbits that crystallization took place in the kidneys of the animals. Preliminary clinical trial seemed to confirm these findings (Sarnoff, 10). The concentrations of the new compound in serum of treated animals were in close agreement to the figures observed with other sulfonamides.

Correspondingly the therapeutic activity of the isoxazole derivative towards representatives of the more important groups of gram-positive and gram-negative pathogenic organisms was quite striking. The values obtained for the minimal (50%) active dose as compared with the figures for other sulfonamides tested in the same experiments showed that Nu-445 was well within the range of activity of the drugs the antibacterial activity of which is clinically well recognized. In the summarizing table 10 the comparative figures of these experi-

ments are given. They make it evident that as a rule 3,4-dimethyl-5-sulfanilamido-isoxazole is of higher activity than sulfathiazole. Its activity is very similar to that of sulfadiazine; small differences in the activity e.g. in infections with streptococci or in infections with type 2 pneumococci are probably still within the range of experimental variation. Only in *Klebsiella* infection did sulfadiazine appear definitely superior.

We are of course aware of the fact that the minimal active doses as determined in animal experiments are only of theoretical importance. A survey of the dosage of different sulfonamides in the treatment of infections of human beings showed the rather surprising tendency of administering the same or even higher doses of more active compounds. The doses recommended for the treatment of pneumonia were frequently larger for sulfathiazole and sulfadiazine than for sulfapyridine despite the fact that sulfadiazine, if tested in mice, was of higher activity than the two other sulfonamides. Similar observations might be made in the treatment of gonorrhea with different sulfonamides. This seemed to suggest that not the minimal active dose but the maximal tolerated dose is considered the guiding principle in clinical chemotherapy.

From this point of view the outstanding properties of solubility and low toxicity particularly for the kidney as offered by 3,4-dimethyl-5-sulfanilamido-isoxazole make this compound appear to be a promising new sulfonamide of therapeutic value.

SUMMARY

1. 3,4-Dimethyl-5-sulfanilamido-isoxazole (Nu-445) and its salts possess remarkably good solubility over a wide pH range particularly in the physiologically important range of pH 6.0-8.0.
2. Due to this solubility no kidney damage was produced after prolonged treatment of rats and rabbits with high doses of the compound.
3. The low toxicity of the new compound and its chemotherapeutic activity in vitro and in experimental infections with the most important representatives of gram positive and gram negative pathogenic micro-organisms indicate that that therapeutic potentialities of the compound compare favorably with those of known and clinically useful sulfonamides.

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THE DIRECT ACTION OF PROSTIGMINE ON SKELETAL MUSCLE; ITS RELATIONSHIP TO THE CHOLINE ESTERS*

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It has been generally stated that prostigmine exerts its pharmacologic action by virtue of its ability to inhibit cholinesterase. However it has been suggested by others on the basis of certain experimental data (1, 2, 3) that in addition to this anti-cholinesterase property it exerts a direct action. The experimental proof of such an action for this drug has not been possible because the anti-cholinesterase effect provided a ready alternative explanation which could not be ruled out.

This report is concerned with the effects of prostigmine and certain choline esters on mammalian skeletal muscle in the absence of cholinesterase. Diisopropyl fluorophosphate (DFP) has been shown to inactivate the cholinesterases irreversibly (4). This agent provided unique experimental conditions for the exploration of the concept of direct action on skeletal muscle by making it possible to carry out experiments on intact muscle which possessed no demonstrable cholinesterase activity; under these conditions the effects obtained were attributed to a direct action of the drug.

METHOD. For this study the intact cat gastrocnemius-soleus preparation was employed. Under Dial anesthesia the Achilles tendon was severed and the gastrocnemius-soleus muscles were freed from all attachments to their origin. The muscles were held vertically by attachment of the tendinous end with cord to an isotonic lever, the origin being fixed rigidly between two metal supports by means of a steel pin driven through the proximal end of the tibia, after the technique described by Wolff and Cattell (5). The popliteal space was exposed and the sciatic trunk and all its branches severed; the artery supplying the muscles was dissected free immediately proximal to them for a distance of about one centimeter. All injections were made as rapidly as possible into this artery with a 26 gauge needle. During the injection the arterial blood supply was temporarily occluded. The volume of fluid injected varied between 0.2 and 0.4 cc. The activity of the muscles was recorded on a smoked drum.

The effect of DFP on the cholinesterase activity of cat skeletal muscle was determined as follows. The gastrocnemius-soleus muscles of one leg were removed and served as controls. The muscles of the other leg were removed 20 minutes after the intra-arterial injection of varying doses of DFP. The muscles were dissected free of all extraneous tissue, cut into small sections, and washed with saline. They were ground with sand in a medium of 0.03 M sodium

*The work described in this paper was carried out under a contract between The Chemical Warfare Service, U. S. Army, and Cornell University Medical College.

bicarbonate (2:1), centrifuged, and the resulting supernatant extract tested for cholinesterase activity by the Warburg manometric technique. In two instances similar determinations were made on serum.

RESULTS. The effect of DFP on the cholinesterase activity of muscle was tested on 7 cats. The results of these experiments are shown in table 1. In each case the cholinesterase activity was reduced to zero following the intra-arterial injection of 0.4 or 1.0 mg./kg. of DFP. Doses of 0.2 mg./kg. of DFP did not abolish completely the cholinesterase activity. All of these doses completely inactivate the cholinesterase of blood (6).

The intra-arterial injection of 0.2 to 1.0 mg./kg. of DFP produced a characteristic response. This response consisted of active, unorganized fasciculations which developed gradually after a latent period of 2 to 5 minutes. A typical response is shown in figure 1. This effect is explainable on the assumption that acetylcholine accumulates after the muscle cholinesterase has been destroyed

TABLE 1
Effect of DFP on muscle cholinesterase activity

CAT NO.	DFP MG/KG BY ARTERY	MUSCLE CHOLINESTERASE ACTIVITY CMM. CO ₂ /HR /1 5 CM.	
		Before DFP	After DFP
25	0.2	54	14
26	0.2	34	12
27	0.2	43	0
28	0.4	35	0
29	0.4	78	0
30	0.4	34	0
21	1.0	116	0

The characteristic contractile response of skeletal muscle to the intra-arterial injection of acetylcholine was found to be unaltered in duration or intensity by the prior administration of DFP. This was confirmed in 4 cats and a typical set of responses is shown in figure 2. Thus the classical response of skeletal muscle to acetylcholine can be effected in the absence of cholinesterase.

The intra-arterial injection of prostigmine bromide or prostigmine methyl-sulfate in a dose of 25 gamma/kg. produced an immediate contraction of the muscle followed by a series of weaker but regular repetitive contractions which persisted for 3 to 4 minutes, differing only in this latter respect from the acetylcholine response. Similar responses were obtained with the prostigmine in 8 cats after the previous intra-arterial injection of 0.4 to 1.0 mg./kg. of DFP; the repetitive contractions in this case were of shorter duration and were followed by cessation of activity. This is illustrated in figure 3. It was possible, therefore, to obtain a response with prostigmine after doses of DFP which were shown to inactivate completely the cholinesterase of the muscle and blood.

To eliminate the possibility that endogenous acetylcholine played a role in these responses the experiments were repeated in 2 cats 7 and 11 days following sciatic nerve section. In each case following the intra-arterial injection of

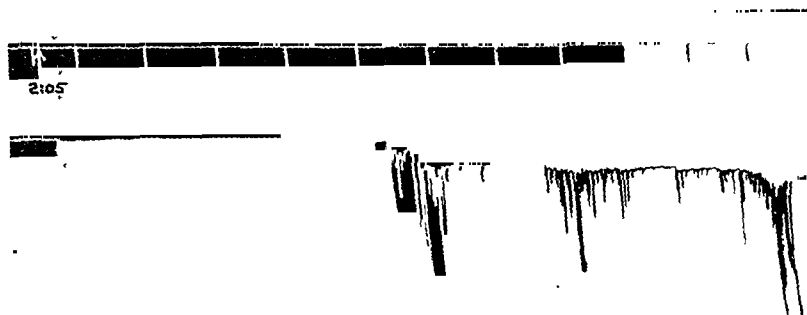


FIG. 1. THE EFFECT OF DFP ON ACTIVITY OF SKELETAL MUSCLE
At 2:05 DFP 1 mg./kg. intra-arterially. Time interval: 30 seconds.

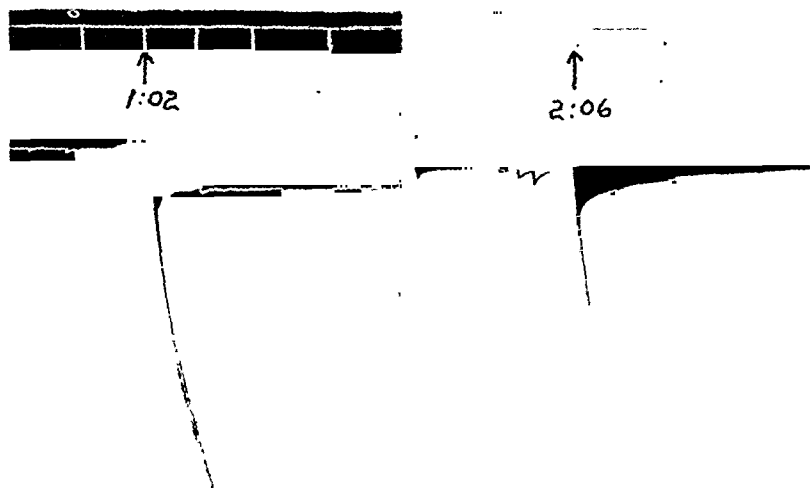


FIG. 2. THE RESPONSE OF SKELETAL MUSCLE TO ACETYLCHOLINE IN
THE PRESENCE AND ABSENCE OF CHOLINESTERASE

At 1:02 acetylcholine bromide 25 gamma/kg. intra-arterially. DFP 1 mg./kg. intra-arterially was given at 1:12. At 2:06 acetylcholine bromide 25 gamma/kg. intra-arterially. Time interval 30 seconds.

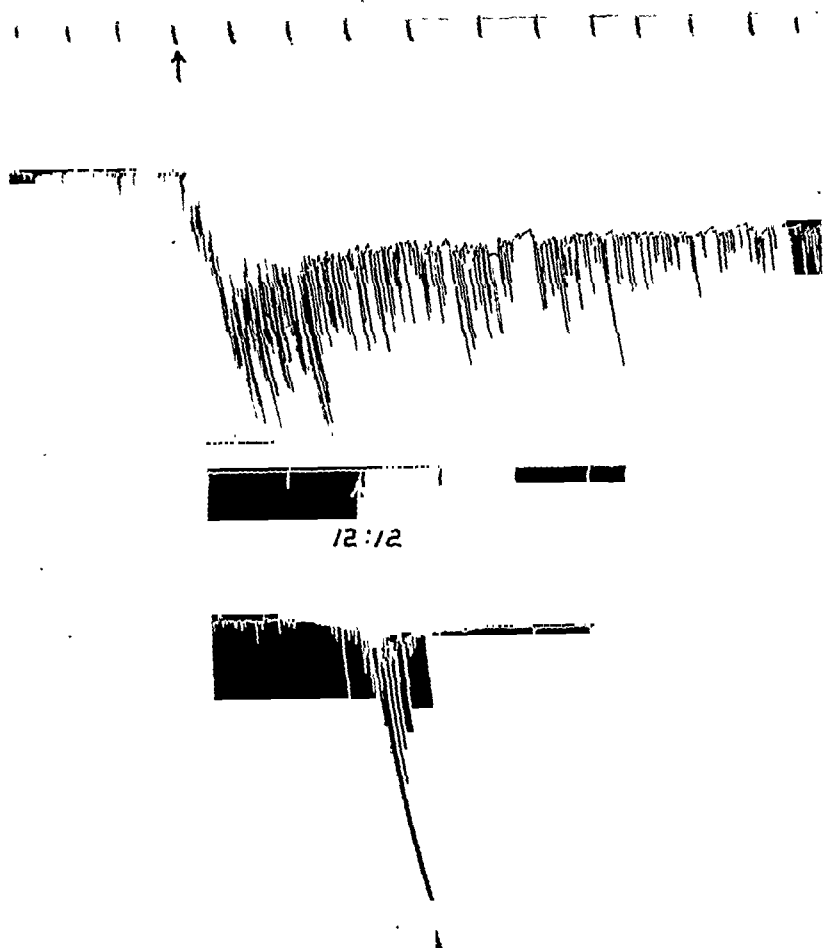


FIG. 3. THE RESPONSE OF SKELETAL MUSCLE TO PROSTIGMINE IN THE PRESENCE AND ABSENCE OF CHOLINESTERASE

Upper tracing: at arrow prostigmine bromide 25 gamma/kg. intra-arterially. Lower tracing: DFP 1 mg./kg. intra-arterially was given at 12:02. At 12:12 prostigmine bromide 25 gamma/kg. intra-arterially. Time intervals: 30 seconds.

1 mg./kg. of DFP the intra-arterial injection of prostigmine bromide produced a response differing only in degree from that obtained with a similar dose of acetylcholine bromide. This response consisted of a prolonged contracture of

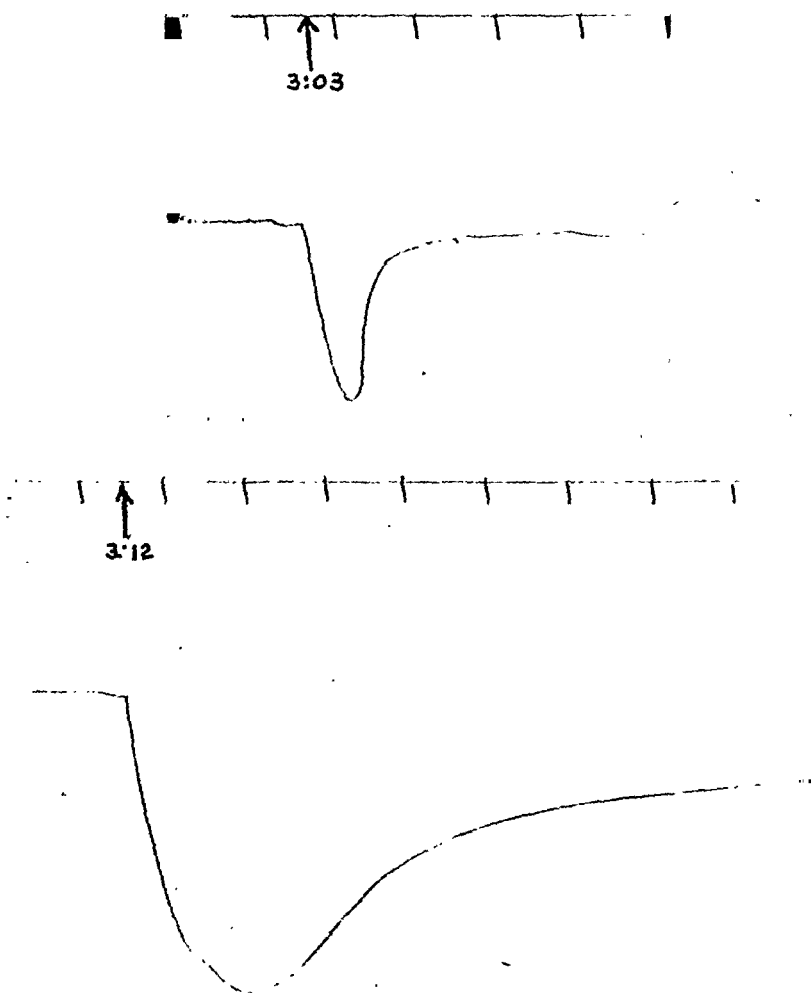


FIG. 4. THE EFFECT OF PROSTIGMINE AND ACETYLCHOLINE ON CHRONICALLY DENERVATED SKELETAL MUSCLE IN THE ABSENCE OF CHOLINESTERASE

Sciatic nerve section 11 days prior to experiment. DFP 1 mg./kg. intra-arterially was given at 2:29. At 3:03 prostigmine bromide 25 gamma/kg. intra-arterially. At 3:12 acetylcholine bromide 25 gamma/kg. intra-arterially. Time intervals: 30 seconds.

the muscle which lasted for several minutes. A typical experiment is shown in figure 4. In these preparations the intra-arterial injection of DFP failed to produce activity in the muscle in contrast to the fasciculations which appeared in

the neighboring muscles which had not been denervated. Since DFP exerts its effects by virtue of its ability to inactivate cholinesterase irreversibly, the lack of activity in the gastrocnemius-soleus muscles following DFP injection provided an excellent demonstration of the absence of acetylcholine.

In 7 cats the repeated intra-arterial administration of single doses of prostigmine bromide (25 gamma/kg.) subsequent to DFP resulted in a progressive diminution of response until the muscle became completely refractory. At this time the muscle also failed to respond to intra-arterial acetylcholine. This unresponsive state of the muscle was produced by an average of 2 injections of the prostigmine. In 2 other animals a single intra-arterial dose of 25 gamma/kg. of acetylcholine following DFP eliminated all base line activity and caused the

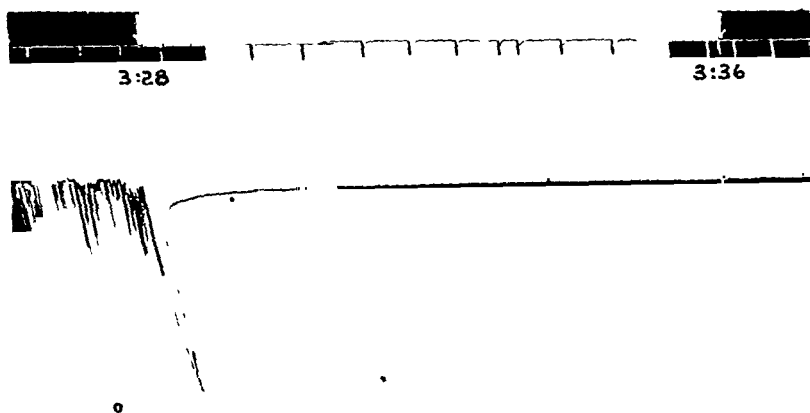


FIG. 5. THE CURARIFORM EFFECT OF ACETYLCHOLINE ON SKELETAL MUSCLE IN THE ABSENCE OF CHOLINESTERASE

DFP 0.4 mg./kg. intra-arterially was given at 3:10. At 3:28 acetylcholine bromide 25 gamma/kg. intra-arterially. At 3:36 prostigmine bromide 25 gamma/kg. intra-arterially. Time interval; 30 seconds.

muscle to become refractory to all subsequent injections. This phenomenon is shown in figure 5.

To illustrate further the relationship between prostigmine and choline esters experiments were carried out with carbaminoylcholine. In 2 cats the response obtained with 47 gamma/kg. of carbaminoylcholine before and after the intra-arterial injection of DFP was similar to but less intense than that obtained with prostigmine. Another point of similarity between prostigmine and carbaminoylcholine has been demonstrated with respect to cholinesterase inhibition. It was shown in 2 human experiments that equivalent doses of each drug produced a like depression of serum cholinesterase activity.

A further analysis of the action of these esters has been made with respect to the correlation of activity with chemical structure. Preliminary experiments have shown that trimethylamine hydrochloride in a dose equivalent to that of

acetylcholine, carbaminoylcholine, and prostigmine produces a similar response (figure 6); this occurs either before or after the administration of DFP. It is evident from this that the trimethylated nitrogen moiety of the choline esters is capable of a direct action on skeletal muscle. On the other hand it has previously been shown by Stedman (7, 8) that the methylcarbamic ester group is the essential feature determining anti-cholinesterase activity. This accounts for the inhibition of cholinesterase activity by prostigmine and carbaminoylcholine.

DISCUSSION. The potency of DFP as an anti-esterase has been well established (4, 9). All the pharmacologic effects of DFP have been attributed to this primary action. The characteristic effects of DFP obtained on the cat muscle

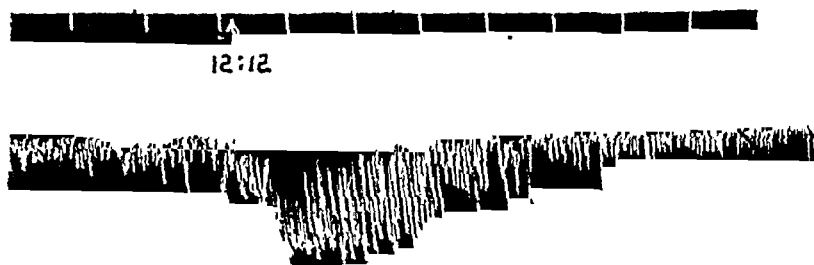


FIG. 6. THE EFFECT OF TRIMETHYLAMINE ON SKELETAL MUSCLE

At 12:12 trimethylamine hydrochloride 25 gamma/kg. intra-arterially. Time interval: 30 seconds.

are in agreement with these findings, in that no immediate contraction occurs and there is a latent period prior to the onset of activity in the acutely denervated preparation; no activity whatever appears in the chronically denervated animal. In both cases the response to prostigmine, which is immediate, is not in accord with that of a primary anti-esterase action. Further, the fasciculations occurring after DFP are irregular and asynchronous in contrast to the regular contractile responses following prostigmine. This latter repetitive response is not due to cholinesterase inhibition since it occurs in the muscle in which the cholinesterase activity has been destroyed by DFP.

It has been pointed out that the classical response of skeletal muscle to the intra-arterial injection of acetylcholine was in no way altered in the absence of esterase. This casts doubt upon the concept that cholinesterase plays a primary role in the mechanism of acetylcholine action.

In our experiments it has been shown conclusively that prostigmine exerts a direct action on skeletal muscle; that is, an action essentially like that of acetyl-

choline. The fact that the response of the muscle is the same in the presence or absence of esterase indicates that the major component of its action is its direct effect; the inhibition of cholinesterase activity by prostigmine is of doubtful significance in this regard. We suggest that the effect of therapeutic doses of prostigmine in myasthenia gravis is due to this direct action on skeletal muscle.

It has been reported previously that excessive amounts of acetylcholine produce a refractoriness in skeletal muscle (10, 11, 12, 13). We have not observed such an effect in the intact animal before the administration of DFP but this acetylcholine phenomenon was elicited after the inactivation of muscle cholinesterase. Prostigmine exerts similar curariform effects on skeletal muscle (14). Our observations confirm this and have shown a corresponding action for carbaminoylcholine. This serves to point out a further resemblance between prostigmine and the choline esters.

The structural formula of prostigmine reveals it to be an analogue of the natural and synthetic choline esters. Since the pharmacologic actions of all these substances are similar it is reasonable therefore to predicate a common chemical basis of action for all rather than to attribute a separate mode of action to prostigmine. The experimental data presented in this paper lend support to this idea, and it is suggested that prostigmine be classified pharmacologically with the choline esters.

The authors are indebted to Dr. McKeen Cattell and Dr. Ephraim Shorr for their helpful suggestions and continued interest.

SUMMARY

1. The destruction of muscle cholinesterase was effected by the injection of an anti-esterase, di-isopropyl fluorophosphate (DFP), into the artery supplying the muscle.

2. The characteristic contractile response of cat skeletal muscle to the intra-arterial injection of acetylcholine was found to be essentially unaltered in the absence of esterase.

3. The effect of DFP on the activity of skeletal muscle differs from that of prostigmine with respect to the time of onset and the contractile character of the response.

4. The response of the muscle to prostigmine, as in the case of acetylcholine, is the same in the presence or absence of cholinesterase.

5. It is concluded that the action of prostigmine is primarily a direct one and differs from that of a primary anti-esterase (DFP).

6. The chemical and pharmacologic similarity of prostigmine, acetylcholine, and carbaminoylcholine is pointed out; it is suggested that prostigmine be classified pharmacologically with the choline esters.

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THE ACTION OF PHYSOSTIGMINE, DI-ISOPROPYL FLUOROPHOSPHATE AND OTHER PARASYMPATHOMIMETIC DRUGS ON THE RECTUS MUSCLE OF THE FROG*

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In a recent report by Riker and Wescoe (1), it was demonstrated that the response of mammalian skeletal muscle to acetylcholine is unaltered in the absence of cholinesterase. The response of striated muscle to neostigmine was also the same in the presence or absence of cholinesterase. On the basis of this evidence it was concluded that the action of neostigmine is direct and differs from that of a primary anti-esterase such as di-isopropyl fluorophosphate (DFP).

The introduction of DFP as an anti-esterase (2) has provided a useful experimental tool for this type of pharmacologic analysis. With the aid of this agent it was possible to test the conclusions of Riker and Wescoe with respect to the action of the cholinergic drugs on the isolated frog rectus preparation. The observations were extended to include an investigation of the mechanism of physostigmine sensitization in the method for acetylcholine assay described by Chang and Gaddum (3).

METHOD. The experiments were carried out on the isolated frog rectus abdominis muscle after the technic of Chang and Gaddum (3) as modified by Nachmansohn (4).

The muscle was held vertically in a chamber of 5 cc. capacity which contained 3 cc. of bicarbonate-buffered frog Ringer's solution. One end of the muscle was attached at the bottom of the chamber and the other connected to a lever recording on a smoked drum. Oxygen was bubbled through the solution continuously at a moderate rate. All experiments were done at room temperature.

The effect of DFP on the cholinesterase activity of the frog rectus muscle was determined as follows: The muscles from ten frogs were soaked for 60 minutes in a DFP solution containing 30 micrograms per cc., washed thoroughly with Ringer's solution for one hour, ground with sand, and centrifuged. The resultant extract was tested for cholinesterase activity using the Warburg manometric technic. The normal cholinesterase activity of untreated muscles was similarly determined. These tests were done on three occasions during the course of the study.

RESULTS. The cholinesterase activity of the DFP treated rectus muscles was reduced to zero.

The effect of DFP on the response of the frog rectus preparation was tested and it was observed that doses in the order of 1.5 mg./cc. were necessary to evoke contraction. Doses of 1 mg. cc. or less failed to produce a response. This

*The work described in this paper was done under contract with the Medical Division, U. S. Chemical Warfare Service.

amount is far in excess of that necessary to inactivate the cholinesterase of the muscle.

To test the effect of DFP on the response of the rectus muscle to acetylcholine five experiments were carried out as follows: A freshly dissected muscle was exposed to acetylcholine (0.1 gamma/cc. for 2 minutes) dissolved in frog Ringer's solution and a small contraction obtained. The muscle was then soaked in the usual concentration of DFP (30 gammas/cc.) for 60 to 90 minutes, thoroughly

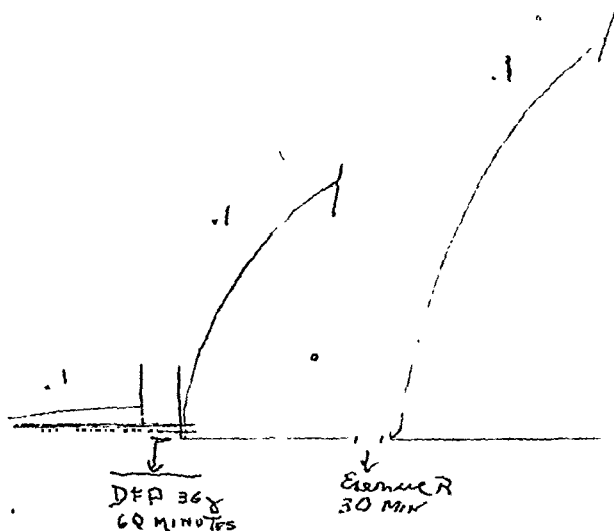


FIG. 1. THE RESPONSE OF A FROG RECTUS PREPARATION TO 0.1 GAMMA/CC. ACETYLCHOLINE

First record: Untreated muscle. Second record: After soaking in DFP (30 gamma/cc.). Third record: After soaking in physostigmine (20 gamma/cc.).

washed out and re-exposed to the same concentration of acetylcholine. The amplitude of the contraction was significantly increased by this treatment. A typical record is illustrated in fig. 1.

To determine the effect of neostigmine, the experiments were carried out as follows: The muscle preparation was exposed successively to increasing doses of neostigmine for periods of ten minutes. The first contractile response occurred at a concentration of 20 gammas per cc. Muscles without cholinesterase activity were also treated with neostigmine (20 gammas/cc.) and contractile responses resulted.

To investigate further the muscle response to neostigmine, seven experiments were carried out as follows: The muscle was exposed to neostigmine and the response was recorded. Then the muscle was soaked in DFP solution as previously described. At this point a second treatment with the same concentration of neostigmine gave an identical response. The ratio between the concentration of acetylcholine and neostigmine giving the same response in DFP treated muscle was found to be about 1:400.

The same technic was employed to determine the action of physostigmine. It was found with this drug that the contractile response was also unaltered after the treatment with DFP; however, larger doses than was the case with neostigmine were necessary. The minimal concentration of physostigmine which produced definite muscle contractions was 0.5 to 1 mg./cc.

In view of the evidence that trimethylamine hydrochloride acts directly on mammalian skeletal muscle (1), the action of this compound was also investigated on the frog rectus muscle. The muscle was exposed to varying concentrations. Definite muscle contraction was obtained with a concentration of 200 gammas/cc. The response of the muscle to trimethylamine was not altered by soaking in DFP solution.

When it was observed that the action of acetylcholine on the frog rectus abdominis muscle was increased by previous treatment with DFP, the phenomenon of potentiation of this response by physostigmine was re-investigated in the following manner: Five experiments were carried out in which the muscles were soaked in DFP solution containing 30 to 40 gammas/cc. for 60 to 90 minutes; the response to acetylcholine was then recorded (0.1 gamma/cc. for 2 minutes). Subsequent to this the muscle was treated with physostigmine Ringer's solution for a period of 35 minutes; the concentration of the drug was 20 gammas/cc. At the end of this time the muscle was rinsed with frog Ringer's solution and re-exposed to the same concentration of acetylcholine. In the DFP treated muscle it was found that the response to acetylcholine was increased, averaging 56 per cent following the exposure to physostigmine. The results from such an experiment are shown in fig. 1.

This procedure for investigating the potentiation of acetylcholine response was tested with neostigmine in five experiments. After the initial acetylcholine response had been obtained in the absence of cholinesterase the muscle was exposed to frog Ringer's solution containing 5 to 10 gammas/cc. of neostigmine. The subsequent acetylcholine response obtained after rinsing with frog Ringer's solution averaged 73 per cent greater.

Similar experiments were carried out with trimethylamine. This compound did not potentiate the response of the muscle to acetylcholine.

DISCUSSION. Concentrations of DFP which were sufficient to inactivate completely the cholinesterase activity of muscles failed to evoke a contractile response. This suggests that endogenous acetylcholine formed by the tissue is insufficient in amount to cause contraction. It may be assumed that the contractions resulting from the large concentration of DFP are due to some other mechanism.

The evidence that DFP increases the response of the muscle to acetylcholine indicates that the inactivation of cholinesterase plays a role in the response of the muscle to acetylcholine, but it is not the entire answer to the question since under these circumstances the use of physostigmine or neostigmine as potentiators provokes a greater muscle response. Furthermore, concentrations of physostigmine which were inadequate to produce muscular contraction were, nevertheless, capable of significantly potentiating the response to acetylcholine at a time when the cholinesterase activity had been destroyed completely by DFP. Therefore the mode of action of physostigmine in potentiating acetylcholine in the usual assay procedure remains to be clarified. In addition to the inhibition of cholinesterase, the drug evidently has a direct action as a potentiator of acetylcholine.

In addition to potentiating the action of acetylcholine, physostigmine in adequate concentration causes contraction of the muscle, which is not prevented by previous treatment with DFP. From this it is concluded that physostigmine stimulates directly the frog rectus muscle.

The results obtained with neostigmine are in line with those reported for its effects on mammalian muscle (1), where the contractile response in the presence or absence of cholinesterase is the same. This is in accord with the concept of a direct action of neostigmine on skeletal muscle. It was observed further that neostigmine, like physostigmine, potentiates the action of acetylcholine in the absence of cholinesterase activity.

Lastly it has been shown that trimethylamine hydrochloride has a direct action on the frog rectus muscle, as is the case with mammalian striated muscle.

The author is deeply indebted to Dr. McKeen Cattell and Dr. Walter F. Riker, Jr. for their constant interest and helpful suggestions without which this publication would not have been possible.

SUMMARY

1. The concept that neostigmine, physostigmine, and trimethylamine act directly on skeletal muscle has been explored on the isolated frog rectus abdominis preparation.

2. Muscles immersed for one hour in frog Ringer's solution containing diisopropyl fluorophosphate (DFP) were shown to possess no cholinesterase activity.

3. The response of the frog rectus muscle to acetylcholine was increased after the inactivation of cholinesterase by DFP.

4. Normal muscles and muscles previously treated with DFP were exposed to suitable concentrations of neostigmine, physostigmine and trimethylamine hydrochloride. Each drug produced contraction which was not influenced by DFP. This indicated that these drugs act directly on muscle.

5. In muscles treated with DFP the response to acetylcholine was augmented by a subsequent treatment with physostigmine or neostigmine.

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THE EFFECTS OF SEVERAL LOCAL ANESTHETICS ON THE RESTING POTENTIAL OF ISOLATED FROG NERVE

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Many anesthetics are more soluble in a lipid than in an aqueous phase and many have a tendency to lower surface tension. These facts have contributed to an understanding of the way in which these substances become concentrated at the cell membrane, their probable site of action. The present study was undertaken with the thought that knowledge of the polar changes occurring at the surface of axones during anesthetic block might furnish clues to the underlying mechanism of such drug action, and, perhaps indirectly, to the functional characteristics of the cell membrane.

Following the observation by Biederman in 1880 (1) that the application of potassium chloride locally on the uninjured frog sartorius caused the surface of the muscle at this site to lose its positive charge and thus become negatively charged in relation to the unaffected surface, the depolarizing action of potassium chloride has been demonstrated repeatedly both on muscle and nerve. Since the difference of potential produced by high concentrations of potassium chloride (17 to 26 times the normal concentration in Maia nerve, Cowan, 1934) (2) corresponds to that between the injured and the intact surfaces of the living cell it has become common practice to use potassium chloride at one electrode in recording the demarcation potential (injury potential, or current of injury). Hereafter we shall use the term demarcation potential to indicate the potential of the intact surface of a living cell in relation to its interior whether contact is made with the interior by actual micro-electrode or as a result of complete local depolarization of the membrane by potassium chloride, heating, or crushing. The term resting potential shall be used to indicate the potential at the surface of any portion of an intact, living cell in relation to the remaining surface of that cell. This normally should be zero at all points on the surface of a cell in equilibrium with its environment. Local changes may cause it to become positive or negative as local polarization respectively increases or decreases under special conditions. Since the reference point for resting potential actually is positive in relation to the interior it may be said that the resting potential increases as it becomes more positive and decreases as it becomes more negative.

During depolarization by potassium chloride there is a progressive decrease in the action potential which disappears before complete depolarization. Both of these effects are completely reversible upon removal of the potassium chloride (2). Bishop in 1932 (3) had observed similar block of the frog sciatic by potassium chloride as evidenced by diminution of the action potential together with progressively developing negativity at the site of application. Up to this time

it was natural to postulate that the depressant drugs cause block of nerve conduction by a similar depolarizing action. However, Bishop found that 0.015 molar cocaine blocked conduction in frog nerve without any evidence of depolarization. In fact he found that it caused a slight increase in the positive potential at the site of application. Likewise he found that amyl alcohol in concentrations from 0.28 to 0.57 molar caused an increase in the resting potential while blocking conduction in frog nerve.

Höber reported in 1939 (4) that 0.0025 molar novocaine caused depolarization of frog muscle and that 0.03 molar concentrations of novocaine and of cocaine also depolarized crab nerve. However he found that 0.03 molar novocaine had no effect on the polarization of frog nerve. He also reported depolarization by amyl alcohol and two urethane derivatives on all three of the above tissues. In view of these apparent discrepancies and the perhaps surprising results obtained by Bishop with two substances, one of which is commonly used for nerve block, it seemed desirable to us that some of these tests be repeated and that the study be extended to a wide variety of local anesthetic drugs.

It is desirable in any such study to follow the course of block by means of the action potential while measuring any changes in the resting potential, thus obtaining evidence not only that the substance is an effective blocking agent and in appropriate concentration for such action but also that it has penetrated to a large number of the fibers of the nerve trunk.

METHOD. Preparation of nerve. The sciatic of the green frog (*R. pipiens*) was dissected from the spinal cord to the heel including the eighth and ninth roots and the peroneal and tibial branches. A silk thread was tied to each end to include the two roots in one and the three branches in the other. All preparation was carried out under a buffered Ringer's solution, and the nerve was kept in this solution until the drugs were applied. Most of the nerves were used the same day. If not used immediately they were refrigerated at 3°C. for periods up to sixteen hours. For the test the nerve was suspended under the electrodes in a moist cabinet previously pictured and described (5). For measurements of demarcation potential 10–12 mm. of the root end was "killed" by the application of a rod heated to 95°C. A glass trough filled with Ringer's solution was then brought up to immerse the entire nerve until the temperature and humidity within the cabinet came to equilibrium. The temperature was kept at 22°C. \pm 0.2° and the humidity at 100%.

Electrodes. Figure 1 shows the positions of the electrodes and anesthetic cup in relation to the nerve. The stimulating electrodes consisted of two silver wires (0.7 mm. diam., spaced 3.5 mm.) and were in contact with the distal, branched end of the nerve. The pick-up electrodes, used for recording all polar changes, consisted of two calomel-Ringer's half-cells each with liquid contact through an orifice 0.5 mm. by 3.0 mm. at right angles to the nerve.

Anesthetic cup. The cup through which the anesthetic solutions were slowly passed (25 cc. per hour) consisted of a glass bulb 1 cm. in diameter into the top of which a V-shaped notch was cut to receive the nerve and the tip of one calomel electrode. The position of the cup could be manipulated from outside the cabinet. The solution entered the cup by dropping into a small funnel lateral to the nerve thus avoiding any continuous column of liquid to act as a conductor from the outside of the cabinet.

Stimulating and recording apparatus. Stimulation of the nerve and the recording of polar changes was carried out in a manner similar to that used by Bishop (3). A rotary rheotome revolving at 10 cycles per second was arranged to provide on-off switching for two independent circuits (see fig. 1). For stimulation and the recording of action potentials

one circuit was used to discharge a 0.1 m.f.d. condenser through a 1000 ohm voltage divider set to deliver one tenth the full voltage to the nerve. The tissue resistance, in parallel with only the 100 ohms, therefore has an insignificant effect upon either the shock duration or voltage. The stimulus thus consisted of condenser discharges of variable voltage at the rate of 10 per sec. and each with a time constant of 0.1 m.sec. (time for 63% discharge).

While recording resting or demarcation potentials one rheotome circuit maintained connection between the pick-up electrodes and the input of the amplifier for periods of 0.08 sec. (0.8 of the complete cycle), thus bringing the amplifier into equilibrium with the potential of the nerve. During the time this circuit was broken the second rheotome contact connected the input of the amplifier to a known source of potential for 0.01 sec. The known potential was adjusted until it matched that of the nerve, using the oscilloscope as a null

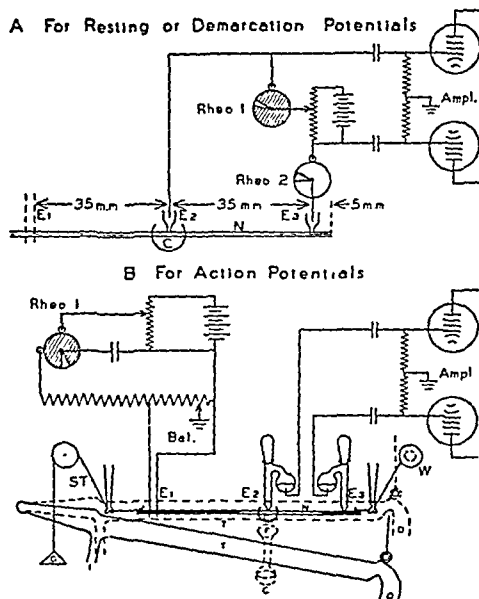


FIG. 1. Ampl., amplifier input. Bal., balancing circuit for escape artefact. C., anesthetic cup. E₁, stimulation electrode. E₂ and E₃, pick-up electrodes. F., funnel inlet to cup. G., 2 gram weight. I., Inlet to trough. N., Nerve. O., Outlet from trough. Rheo. 1 and 2., Rheotome. S.T., Silk thread. W., Windlass.

point indicator. The amplifier-sensitivity was attenuated to 5 mm. displacement on the oscilloscope screen per millivolt at the nerve.

A resistance-capacity coupled amplifier with balanced input was used both for recording action potentials and for measuring resting and demarcation potentials. The circuit was push-pull from input to the deflection plates of the oscilloscope. Maximum sensitivity was 5 microvolts per millimeter. The time constant was 0.06 sec.

A Dumont Oscilloscope, Type 175-A, was used with a Dumont Low Frequency Time Base Generator, Type 215, as the sweep circuit. The linear velocity of sweep was 10 mm. per m.sec., and a single sweep was tripped by the rheotome 10 times per sec.

Solutions. The Ringer's solution contained per liter: NaCl, 6.5 grams; KCl, 0.1 gram; CaCl₂, 0.1 gram; Na₂HPO₄ (12 H₂O), 1.92 grams; and NaH₂PO₄ (1H₂O), 0.184 gram. The pH was from 7.2 to 7.4.

All the anesthetic solutions were made up to the designated molarity in the same Ringer's solution. The following formulae indicate the variety of agents studied:

1. Cocaine Benzoyl methylec-
gonine

$$\begin{array}{c} \text{H}_2\text{C}-\text{CH}-\text{CH CO}_2\text{CH}_3 \\ | \quad | \quad | \\ \text{NCH}_3 \quad \text{CHOO C} \text{ (cyclohexyl)} \\ | \quad | \quad | \\ \text{H}_2\text{C}-\text{CH}-\text{CH}_2 \end{array}$$
2. Procaine (hydrochloride) Diethylamino ethyl
para amino benzoate

$$\text{NH}_2 \text{ (cyclohexyl)} \text{CO}_2\text{CH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2\text{HCl}$$
3. Intracaine β -diethylamino ethyl
4-ethoxy benzoate

$$\text{C}_2\text{H}_5\text{O} \text{ (cyclohexyl)} \text{CO}_2\text{CH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2$$
4. Borocaine Ethocaine borate

$$\text{NH}_2 \text{ (cyclohexyl)} \text{CO}_2\text{CH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2 \cdot 5\text{HBO}_2$$
5. Monocaine Mono iso-butylamino
ethyl para am no
benzoate hydro-
chloride

$$\text{NH}_2 \text{ (cyclohexyl)} \text{CO}_2\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{HCl}$$
6. Pontocaine *p*-butylamino benzoyl
dimethylamino
ethanole

$$\text{C}_4\text{H}_9\text{NH}_2 \text{ (cyclohexyl)} \text{CO}_2\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$$
7. Metycaine α (2 methyl piperidino)
propyl benzoate

$$\text{(cyclohexyl)} \text{CO}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N} \begin{array}{c} \text{CH}_3 \\ | \\ \text{CH}-\text{CH}_2 \\ | \quad \diagup \\ \text{CH}_2-\text{CH}_2 \end{array}$$
8. Nupercaine α -butyl oxycincho-
nic acid diethyl-
ethylene diamide

$$\begin{array}{c} \text{CONHCH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2 \\ \text{(naphthalene ring)} \\ \text{OC}_4\text{H}_9 \\ \text{N} \end{array}$$
9. Naphthacaine β -diethylamino ethyl
ester of 4-amino-L-
naphthoic acid
mono hydrochlo-
ride

$$\begin{array}{c} \text{CO}_2\text{CH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2 \cdot \text{HCl} \\ \text{(naphthalene ring)} \\ \text{NH}_2 \end{array}$$

10. γ -phenyl- γ -hydroxy- β (diethylamino) propyl benzoate
- CCN(CC)CC(O)C(=O)C1=CC=CC=C1
11. β -diethylamino ethyl trans α ethyl cinnamate
- CCN(CC)CCOC(=C)C1=CC=CC=C1
12. β -(N-methyl-N-phenethylamino) ethyl carbanilate
- CCN(C)CCOC(=O)C1=CC=CC=C1
13. γ -diethylamino propyl phenyl urethane
- CCN(CC)CCOC(=O)C1=CC=CC=C1
14. β Eucaine lactate
- CCN(CC)CCOC(=O)C1=CC=CC=C1

PROCEDURE. After the temperature and humidity of the cabinet had become stable, during which time the nerve was immersed in Ringer's solution, the trough was dropped to expose the nerve. Five minutes later the resting or demarcation potential was determined depending upon whether or not the end of the nerve had been heat-treated. This determination was repeated at 10 minute intervals until at least two consecutive readings showed the same potential. During the same control period the irritability of the nerve was checked by determining the minimal effective stimulation voltage and the voltage necessary to produce the maximal A α wave of action potential. Using twice this maximal stimulus the amplifier was adjusted to give an A α wave of 50 mm. on the screen. Percentage block as shown in column 3 of table 1 was considered simply as the percentage decrease in this wave height.

When action potential and resting or demarcation potential were constant the cup containing the anesthetic solution was raised to envelope the nerve and the tip of electrode E₁ within the meniscus of the solution. The solution was dropped slowly through the cup at a rate of 25 cc. per hour throughout the experiment. This was continued for periods from 7 to 100 minutes while the degree of block was followed by the decrease in action potential. Resting or demarcation potentials were determined from time to time during this period. After various degrees of block (from 44 to 95% in terms of diminished action potential) the cup was dropped and the trough raised to wash the nerve three times in Ringer's solution

after which the nerve was exposed for a recovery period during which potential readings were continued.

CONTROLS. Twelve nerves were followed for periods from one half to three hours without applying any solutions or, in some cases, applying Ringer's solution in the cup in order to establish the stability of the resting potential or the rate of decline in the demarcation potential under the conditions of these experiments. It was found that when the initial resting potential (5 minutes after exposure of the nerve) was less than 1.5 mv. it remained constant within 0.5 mv. over several hours. One nerve was followed for more than eleven hours while the resting potential changed 0.2 mv. In evaluating the significance of this change it should be related to the full, recordable potential of the nerve by the demarcation

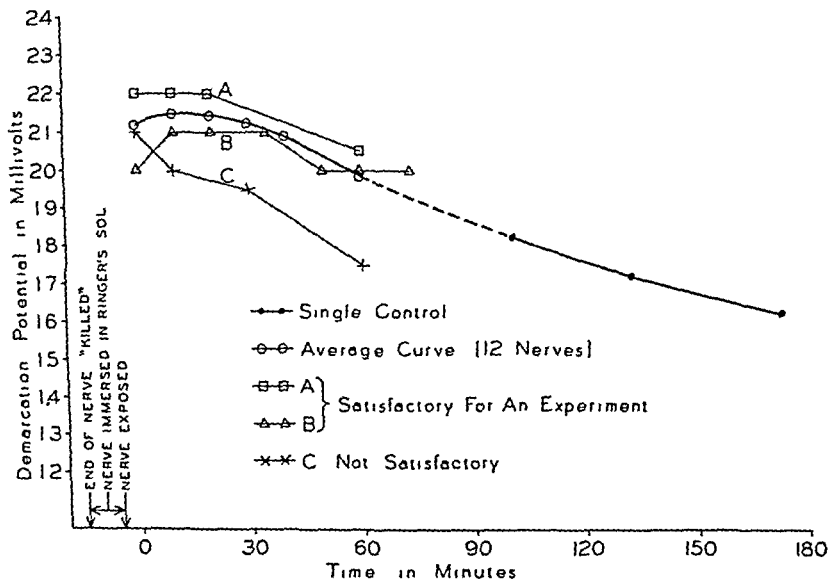


FIG. 2. CHANGES IN DEMARCATION POTENTIAL OF FROG SCIATIC NERVE EXPOSED TO AIR AT 22°C., SATURATED WITH WATER VAPOR

method. This averaged 21.5 mv. in these experiments. Since an initial resting potential of more than 1.5 mv. was considered as evidence of damage to the nerve such nerves were discarded.

Figure 2 shows a curve of the average changes in demarcation potential over a period of one hour as observed in twelve control experiments. This curve is continued with the data from one nerve to cover a total of nearly three hours. With one exception (No. 6) no drug was applied during an experimental period to exceed 40 minutes. Along with the average curve are shown three curves from selected controls to illustrate (e.g. A and B) nerves which were considered to meet the requirement (stated under Procedure) of a ten minute period of isopotential before adding a drug and (e.g. C) a nerve which would not be used because of its failure to meet this requirement. The average curve was used to determine the expected fall of potential during each experimental period as indicated in column 7 of table 1. In view of the individual variations from the average, no change of potential of less than 1 mv. during an experimental period was considered significant.

Bathing the nerve locally through the cup with Ringer's solution instead of anesthetic in no way affected the potential readings.¹

RESULTS. Results are presented in table 1. In column 6 are the observed changes in resting and demarcation potential. In column 8 are the changes in demarcation potential corrected for expected change during the time of the experiments as estimated from the average control curve.

TABLE 1
Effects of anesthetics on demarcation and resting potentials

	1	2	3	4	5	6	7	8
	CONCENTRATION	PERIOD DRUG APPLIED	BLOCK	POTENTIAL BEFORE DRUG	POTENTIAL AFTER DRUG	OBSERVED CHANGE IN POTENTIAL	EXPECTED CHANGE DURING SIMILAR CONTROL PERIOD	CHANGE IN POTENTIAL DUE TO DRUG
	<i>m. Mol.</i>	<i>minutes</i>	<i>per cent</i>	<i>m.V.</i>	<i>m.V.</i>	<i>m.V.</i>	<i>m.V.</i>	<i>m.V.</i>
Demarcation potentials								
1. Cocaine	4	30	90	20	20	0	-0.9	+0.9
2. Procaine .. .	10	24	86	23	22	-1	-1.0	0
3. Intracaine. . .	1.5	40	58	21	20	-1	-1.1	+0.1
4. Borocaine. . .	5	7	80	23	23	0	-0.3	+0.3
5. Monocaine . .	10	14	84	23	22	-1	-0.7	-0.3
6. Pontocaine	0.1	100	78	19	18	-1	-3.8	+2.8
7. Metycaine	5	11	90	20	20	0	-0.4	+0.4
8. Nupercaine.. .	1	40	90	20	19	-1	-1.8	+0.8
9. Naphthacaine ..	1	15	90	23	22	-1	-0.7	-0.3
10. *	0.1	20	44	21	21	0	-0.1	+0.1
11. * ..	5	8	74	22	21	-1	-0.3	-0.7
12. * ..	1	5	70	23	22	-1	-0.3	-0.7
13. * ..	5	16	86	21	21	0	-0.6	+0.4
Resting potentials								
1. Cocaine	4	20	60	1.2	1.3	+0.1		+0.1
2. Procaine ..	5	35	50	1.3	2.0	+0.7		+0.7
	10	20	86	1.5	2.2	+0.7		+0.7
6. Pontocaine	0.25	14	92	1.2	1.6	+0.4		+0.4
14. *	10	11	95	0.5	0.6	+0.1		+0.1

* Chemical names and formulae listed on page 75.

Note that in no instance was there a drop in either resting or demarcation potential (column 6 or 8) in excess of 1 mv. although there is evidence in all cases that a considerable number of nerve fibers have been blocked. Two of the drugs, when permitted to act for longer periods of time, caused a drop in potential. Number 11 caused a drop of 4.2 mv. (corrected as in column 8) while

¹ It was observed, however, that immersion of the whole nerve in Ringer's solution between readings caused the demarcation potential to decline at the rate of about 6 mv. per hour instead of from 2 to 2.5 mv. as in the exposed nerve after the first 30 minutes.

blocking 92 per cent in 20 minutes. Number 12 caused a drop of 1.5 mv. while blocking 94 per cent in 12 minutes. Since these drugs caused as much as 74 and 70 per cent block, respectively, without significant change in demarcation potential, the change observed during prolonged action was not considered essential to the mechanism of block.

In the case of pontocaine, although the observed change in potential was 1 mv., when this was corrected for the unusually long period the drug was applied it appeared that the drug had caused a rise in potential of 2.8 mv. This illustrates a general impression which developed concerning many of the drugs, namely that, although they caused no significant change in the potential either way, they tended to stabilize the demarcation potential during the time of application and that this potential then dropped more rapidly than usual after removal of the drug. Since the decline in demarcation potential of the exposed, untreated nerve is evidently due to changes at the junction of "killed" and living tissue ("crush sealing over" effect, Gerard) (6) because the potential can be restored by reheating this junction, we feel that this apparent stabilizing effect of the drugs is, rather, a true tendency toward higher positive potentials at the site of block. This was probably exaggerated in the case of the effect of 0.1 millimolar pontocaine on demarcation potential because of the long period the drug was applied and the correspondingly large correction based upon the expected fall during this period. In another experiment 0.26 millimolar pontocaine caused the resting potential to increase only 0.4 mv. while blocking 92% during a period of fourteen minutes.

DISCUSSION. The evidence presented establishes that a considerable variety of chemical agents, at proper concentrations, block conduction in a large number of fibers within a nerve trunk without depolarization. Thus Bishop's conclusion based on his findings with amyl alcohol and cocaine has been confirmed.

Höber's observations (4) of depolarization by cocaine, procaine, and amyl alcohol need not be considered incompatible with the results of Bishop (3) and with those here presented. The concentrations used by Höber were greater than those necessary to block conduction. He used on crab nerve a concentration of cocaine (30 millimolar) which was twice that used by Bishop and more than seven times that with which we produced 90 per cent block in frog nerve. In the same paper Höber presented evidence to indicate that crab nerve is more sensitive to drug effects than is frog nerve. He also used a concentration of amyl alcohol (250 millimolar) which was almost twice that used by Bishop to produce approximately 80 per cent block. In the case of procaine Höber found no depolarization of frog nerve with a concentration (30 millimolar) which was three times that with which we produced 86 per cent block. We have reason to believe from our own observations as well as from the experiments of Höber that several of the anesthetics in sufficiently high concentrations and when applied for prolonged periods will depolarize nerve. These observations, without corresponding data concerning the degree of block and the time during which the drug has been applied (Höber presents neither), reveal nothing of the mechanism of block.

The evidence that conduction of impulses is prevented by anesthetics without depolarization of nerve forces us to the alternate concept, namely that the anesthetic tends to fix the conditions about the cell membrane thus preventing the phasic shifts normally associated with conduction. This is consistent with the evidence presented by many (7, 8, 9, and 10) that anesthetic block is associated with decreased permeability of the cell membrane. We are further encouraged in this belief by our recent observation that the shift in resting potential which is consistently produced by a change in calcium concentration is prevented by previous treatment of a nerve with procaine (not published).

If this change in permeability of the cell membrane were to be considered the essential cause of block, the increase in threshold for stimulation and the increased duration of the refractory period which we and several others have observed at the site of incomplete anesthetic action may reasonably be ascribed to the same cause. The increase in the refractory period, which may be thought of as a decrease in the rate at which normal relationships are reestablished about the less permeable membrane, easily accounts for the observation that many anesthetics block the conduction of impulses at high frequency before the stage of complete block.

We believe that the more stable preparation used in measuring resting potentials of nerve in contrast to that for demarcation potentials justifies the continued use of the former procedure. Although the demarcation method provides the possibility of relating potential changes to the full, recorded potential of the cell or cell aggregate, this relationship can hardly be considered quantitatively significant as it is measured in a compound structure such as a nerve. It is therefore considered of questionable advantage.

SUMMARY AND CONCLUSIONS

By a study of resting and demarcation potentials in isolated frog sciatic nerves it has been shown that a variety of anesthetic drugs block conduction without depolarizing the nerves.

These findings extend the significance of Bishop's observation concerning cocaine and justify the conclusion that anesthetics in general block nerves because they stabilize those conditions, relative to the cell membrane, which normally are sufficiently labile to permit the phasic shift in potential during impulse conduction.

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THE BIOCHEMORPHOLOGY OF NICOTINE

I. OBSERVATIONS ON THE EFFECT OF PROGRESSIVE DEGRADATION OF THE PYRROLIDINE RING

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During the course of our studies on the fate of nicotine in the body (1, 2), we have had occasion to make observations on the pharmacologic actions of a number of nicotine derivatives involving progressive degradation of the pyrrolidine ring. Through subsequent addition to these, we have arrived at the rather complete series diagramed in figure 1.¹

Observations similar to those we will report have been published for several of the members in this series and hence no claim to priority of observation on any given member should be inferred. However, since in a correlation of this type uniformity of technique and observation is of very considerable importance, only data personally obtained have been used.

The observations and correlations that we have made follow.

EXPERIMENTAL. All toxicity studies reported were made on mice. At least 40 mice were employed in obtaining each intraperitoneal LD₅₀ value, the minimum number being 20 at each of two significant points on either side. Similarly, at least 20 mice were used for each intravenous LD₅₀ value. Concentrations used in these toxicologic studies were such that the intraperitoneal dose volumes were 20 cc. per kgm. and the intravenous dose volumes were 5 cc. per kgm. body weight. In the mice receiving intraperitoneal injections, the time of death and the intensity of the characteristic response were also noted.

The blood pressure studies were made on dogs under Dial anesthesia. Those substances showing pressor activity were compared for potency on the basis of the dose required to effect a blood pressure rise equivalent to that produced by 0.00012 mM (0.02 mgm.) per kgm. of nicotine administered intravenously. Those substances showing depressor activity were compared on the basis of minimum effective dose. Three dogs were used in assaying each substance. Where variations were small the reported values are based on averages; where variations were large, ranges are indicated.

RESULTS. The data obtained are summarized in table 1, the order of presentation of compounds being similar to that followed in figure 1.

Effect of demethylation. The effect of demethylation is apparent in the following five pairs of compounds: nicotine vs. nor nicotine, 3-(4-methylaminobutyl)-

¹ We are indebted to Dr. R. C. Roark, U. S. Department of Agriculture, Bureau of Entomology and Plant Quarantine, for the nor nicotine used in these studies; to Dr. C. F. Woodward, Eastern Regional Research Laboratory of the U. S. Department of Agriculture, for the 3-(4-aminobutyl)-pyridine, and to Dr. C. H. Rayburn and Mr. H. N. Wingfield of the Research Laboratory of the American Tobacco Company, for the N-methyl butylamine, pyrrolidine, N-methyl pyrrolidine, 3-(4-methylaminobutyl)-pyridine, 3-(1-methylaminobutyl)-pyridine, 3-(1-aminobutyl)-pyridine, 3-(1-aminoethyl)-pyridine and 3-butyl pyridine.

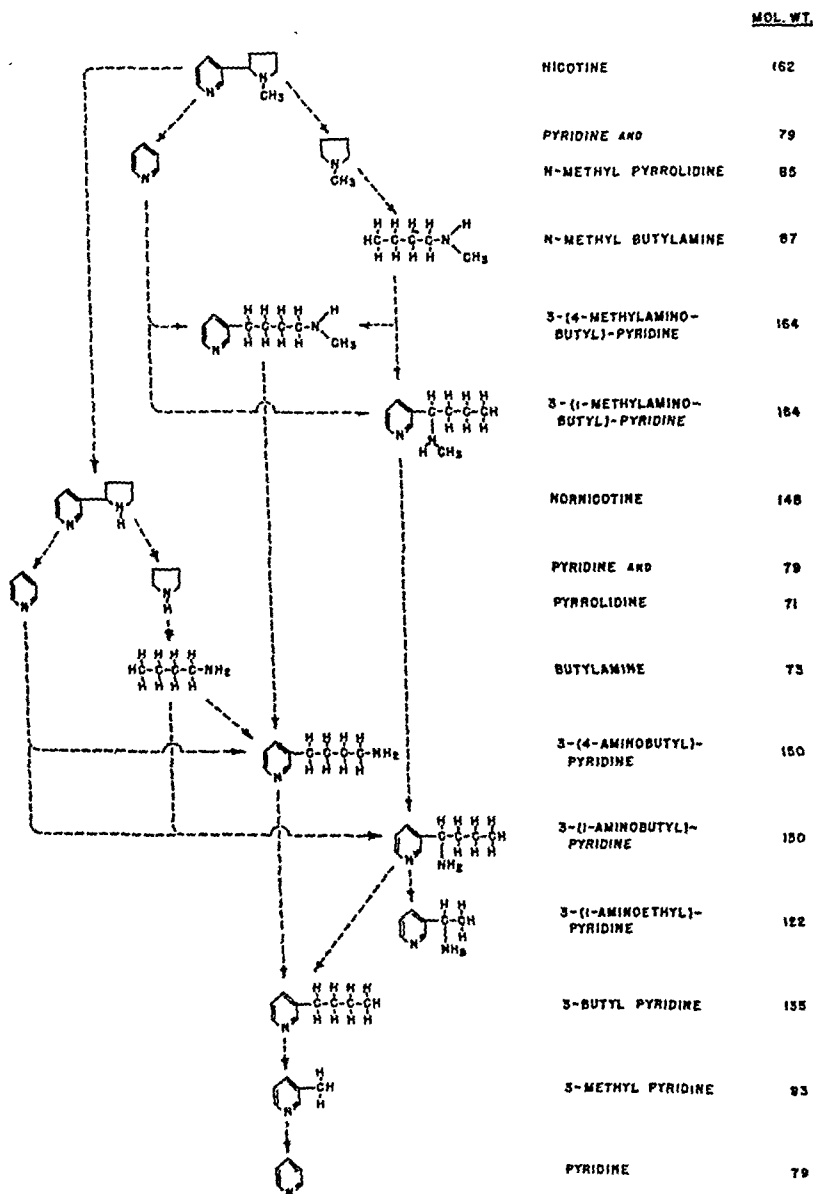


FIG. 1

pyridine vs. 3-(4-aminobutyl)-pyridine, 3-(1-methylaminobutyl)-pyridine vs. 3-(1-aminobutyl)-pyridine, N-methyl pyrrolidine vs. pyrrolidine, N-methyl butylamine vs. butylamine.

In each case, demethylation decreased the acute toxicity whether the compound was given intraperitoneally or intravenously. An exception to this has been previously noted by us in the rabbit (3) for which nornicotine is more toxic

TABLE 1

SUBSTANCE	LD ₅₀ , mM PER KG. (MOUSE)		I.P. /I.V.	RELATIVE TOXICITY EQUIMOL. BASIS		AVE. TIME OF DEATH (I.P.) MINUTES	B.P. EFFECT (DOG I.V.)		CNS EFFECT	
	I.P.	I.V.		I.P.	I.V.		Pressor (Dose equiv. to 0.00012 mM nicotine per kgm.)	De- pressor (Thres- hold dose) mM per kgm.	Convul- sant	Arres- thet- ic
Nicotine.....	0.064	0.0034	19	100	100	2.9	0.00012		++	
N-methyl pyr- rolidine	2.1	0.55	3.8	3.0	0.62	7.8	0.035	0.0018	+	
N-methyl butyl- amine.....	5.4	1.4	3.8	1.2	0.24	11		0.0029	++	
3-(4-methyl- amino-butyl)- pyridine.....	0.64	0.070	9.1	10	4.9	2.7	0.0037		+	
3-(1-methyl- amino-butyl)- pyridine.....	0.76	0.24	3.2	8.4	1.4	7.0	0.055		+	
Nornicotin.....	0.15	0.023	6.5	43	15	6.5	0.0016		+	
Pyrrolidine.....	5.9	1.2	4.9	1.1	0.28	13	0.016	0.0021	+	
Butylamine.....	8.6	2.7	3.2	0.74	0.13	12	0.027-0.19		+++	
3-(4-amino- butyl)-pyri- dine.....	1.5	0.52	2.9	4.3	0.65	17	0.0027		+	
3-(1-amino- butyl)-pyri- dine.....	2.3	0.54	4.3	2.8	0.63	8.9		0.0057	+	
3-(1-amino- ethyl)-pyridine	3.9	1.3	3.0	1.6	0.26	11	0.041-0.066		+	
3-butyl pyridine	2.0	0.44	4.5	3.2	0.77	13		0.00037		+
3-methyl pyri- dine.....	6.4	3.2	2.0	1.0	0.11	See text		0.032		+
Pyridine.....	14	6.8	2.1	0.46	0.050	See text		0.013		+

than nicotine by intravenous injection. However, the rabbit is not consistent in this respect, for we have found for it that N-methyl pyrrolidine is more toxic than pyrrolidine.

Survival time in mice following intraperitoneal injection of fatal doses was uniformly increased by demethylation.

Concerning pressor potency, a decrease in potency through demethylation occurred only in the cases of nicotine vs. nornicotine and 3-(1-methylaminobutyl)

-pyridine vs. 3-(1-aminobutyl)-pyridine. In the other three pairs demethylation increased pressor potency. From this it would appear that in this series the presence of the pyridine component determines whether or not demethylation will increase or decrease pressor potency with the further stipulation that for decreased pressor potency on demethylation the amino group must be attached to the first carbon in the side chain or ring.

Effect of opening the pyrrolidine ring. The effect of opening the pyrrolidine ring is shown in the following groups of compounds: nicotine, 3-(4-methylaminobutyl)-pyridine, 3-(1-methylaminobutyl)-pyridine; nornicotine, 3-(4-aminobutyl)-pyridine, 3-(1-aminobutyl)-pyridine; N-methyl pyrrolidine, N-methyl butylamine; pyrrolidine, butylamine.

In each case opening the pyrrolidine ring at either of the N-C linkages decreased the acute toxicity whether the compound was given intraperitoneally or intravenously. In addition, in the cases of nicotine and nornicotine the decrease in toxicity is greatest when the pyrrolidine ring is split between the nitrogen and the 5 position.

Concerning pressor potency, opening of the pyrrolidine ring decreased and in some instances [3-(1-aminobutyl)-pyridine, N-methyl butylamine] even reversed pressor activity. In the case of the nicotine and nornicotine derivatives, decrease in pressor potency is greatest when the amino group is attached to the first carbon in the side chain.

Effect of position of the amino group. Effect of position of the amino group in the case of the open side chain derivatives is apparent in the compounds 3-(4-methylaminobutyl)-pyridine, 3-(1-methylaminobutyl)-pyridine, 3-(4-aminobutyl)-pyridine, 3-(1-aminobutyl)-pyridine.

Both acute toxicity and pressor potency are greatest when the amino group attachment is to the 4 position in the side chain. Yet it is apparent in the case of nicotine and nornicotine that simultaneous substitution of the same amino group in both positions (cyclization) tremendously increases toxicity and pressor potency.

Effect of deamination. The most striking effect of deamination is the complete reversal in the toxic manifestations. In all instances, the presence of an amino group led to toxic symptoms that were convulsant in character in contrast to the primary anesthetic action of 3-butyl pyridine, 3-methyl pyridine and pyridine itself.

Pyridine, 3-methyl pyridine and 3-butyl pyridine all caused a fall in blood pressure with pyridine occupying an intermediate position with regards to depressor potency.

Effect of shortening the side chain. Observation in this connection were limited to a comparison of 3-(1-aminobutyl)-pyridine vs. 3-(1-aminoethyl)-pyridine and of 3-butyl pyridine vs. 3-methyl pyridine.

In each case, shortening of the side chain resulted in a decrease in acute toxicity.

As regards blood pressure effects, shortening of the 1-aminobutyl side chain to 1-aminoethyl converted the substance from a depressor to a pressor one, and

shortening of the butyl side chain to methyl reduced the depressor activity of the substance about ninety-fold.

Miscellaneous observations. Like epinephrine, N-methyl pyrrolidine and pyrrolidine may exert either depressor or pressor effects on blood pressure depending on the dose administered. At their threshold of effectiveness they produce purely depressor responses. The dose range for this effect is 0.0018–0.012 mM (0.15–1.0 mgm.) per kgm. for N-methyl pyrrolidine and 0.0021–0.0070 mM (0.15–0.5 mgm.) per kgm. for pyrrolidine. Above these ranges the responses become biphasic, a rise following the fall, and with increasing doses the rise in blood pressure becomes increasingly predominant.

With regard to survival time following intraperitoneal administration of 3-methyl pyridine and pyridine, the ranges observed were too wide to apply averages in the sense used for the other substances reported. The majority of deaths in the case of 3-methyl pyridine were early (average 13 minutes). However, several deaths were considerably delayed ($\frac{1}{2}$ to 4 or more hours). With pyridine, about half of the deaths were early (average 9 minutes), the remainder being more delayed ($\frac{1}{2}$ to 4 or more hours).

SUMMARY

The effect of progressive degradation of the pyrrolidine ring of the nicotine molecule on acute toxicity (mouse) and blood pressure (dog) has been studied.

Acute toxicity was decreased by:

(a) N-demethylation.

(b) Opening of the pyrrolidine ring between the N-C linkage, the decrease being greatest when the cleavage occurred between the nitrogen and the 5 position.

(c) Shortening of the resultant side chain following N-C cleavage between the 1 and 5 positions.

Furthermore, in all instances reported, deamination altered the characteristic response from a convulsant one to an anesthetic one.

Pressor potency was decreased by:

(a) N-demethylation provided the nitrogen was linked to the first carbon of a beta substituted pyridyl molecule; in all other pairs studied demethylation increased pressor potency.

(b) Opening of the pyrrolidine ring between the N-C linkage, the decrease being greatest when the cleavage occurred between the 1 and 5 positions. In the latter case, shortening of the resultant side chain increased pressor potency.

(c) Deamination. All deaminated derivatives studied were depressor and those compounds that were originally depressor despite the presence of an amino group became more so on deamination.

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THE METABOLIC FATE OF ACETANILID AND OTHER ANILINE DERIVATIVES

I. MAJOR METABOLITES OF ACETANILID APPEARING IN THE URINE

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Shortly after the introduction of acetanilid into therapy in 1886 (1) it was recognized that this drug and other aniline derivatives undergo metabolic changes in the body. The attempt to elucidate these changes has, in the intervening years, been characterized more by extensive theorizations than by experimental demonstrations. The present series of papers, of which this is the first, reviews these theories and, with newly developed methods for analytical determination of possible metabolites, subjects them to experimental review. The present paper deals with the major metabolites appearing in the urine.

In 1887, Cahn and Hepp (2) reported that after acetanilid was given to dogs only a part appeared unchanged in the urine. Della Cella (3), in the same year, using men, found none in the urine. A year later Jaffe and Hilbert (4) also reported that, contrary to Cahn and Hepp, no acetanilid could be found in the urine of dogs but that aniline derivatives were present; these they identified as p-aminophenol conjugated with sulfuric and glycuronic acids and as conjugated 2-benzoxazolone. These same investigators pointed out that the fate of acetanilid differs in herbivorous and carnivorous animals, for in rabbits given acetanilid they found only conjugates of p-aminophenol in the urine. The occurrence of p-aminophenol as a sulfate or glycuronate or as N-acetyl-p-aminophenol in the urine after administration of acetanilid to men and animals has been abundantly confirmed (5-12).

There is little experimental data reported concerning the occurrence of any metabolites of acetanilid in body fluids other than urine. Payne (9) and Young and Wilson (10) reported the identification of p-aminophenol in the blood of dogs after administration of acetanilid but they did not determine the amount or whether it was free or conjugated. Ellinger (13) extracted from the pooled blood of four cats which had received acetanilid a small amount of a material which had the physico-chemical properties of N-acetylphenylhydroxylamine.

Although the presence of free aniline had never been demonstrated in the blood after administration of acetanilid, Michel, Bernheim and Bernheim (14) reported that *in vitro* tissue suspensions of the rat could hydrolyze acetanilid.

These limited findings concerning the end products of acetanilid excreted in the urine and the much more meager data concerning metabolites occurring in the blood have been the sole direct evidence to support the numerous and conflicting theories of the metabolism of acetanilid. These theories were postulated, to a large extent, on the basis of the *in vitro* chemistry of acetanilid and related compounds and upon observations of the methemoglobinemia and the

toxicological and pharmacological effects following administration of possible intermediary metabolites.

Thus Cahn and Hepp (2) postulated that the part of the acetanilid which was not recovered in the urine in their experiments was split into aniline and acetic acid in the body. Jaffe and Hilbert (4) were unable to confirm this, but, finding mainly conjugated 2-benzoxazolone in the urine of dogs after acetanilid, they hypothesized the following metabolism of acetanilid for carnivorous animals: acetanilid is oxidized to oxyphenylcarbamic acid which, being unstable, loses water to form 2-benzoxazolone which, in turn, is conjugated with sulfuric or glycuronic acid and excreted in the urine. Since these investigators found only p-aminophenol in the urine of rabbits given acetanilid they concluded that in herbivorous animals the acetyl group disappears and p-aminophenol is formed, which is then conjugated with sulfuric or glycuronic acid and excreted.

Mörner (7), on the basis of the occurrence of N-acetyl-p-aminophenol conjugated with sulfuric acid in the urine of men who had been given acetanilid, concluded that acetanilid is oxidized to N-acetyl-p-aminophenol, which is then conjugated and excreted.

In 1913 Heubner (6) expressed the opinion that the methemoglobinemia resulting from aniline and its derivatives might be due to the p-aminophenol formed during metabolism and he therefore studied the quantitative action of this substance as a methemoglobin former in cats, dogs and rabbits. He found that each molecule of p-aminophenol given resulted in the formation of many molecules of methemoglobin. He theorized a cyclic reaction in the body in which p-aminophenol is oxidized to quinone imine. The latter oxidizes hemoglobin to methemoglobin and is thereby reduced to p-aminophenol. Heubner concluded that p-aminophenol is the only important metabolite of aniline and its derivatives.

Lipschitz (15), in 1920, disagreed with Heubner's contention of a cyclic reaction of p-aminophenol with the formation of a quinone and postulated a different main metabolite of aniline and its derivatives for the formation of methemoglobin. He assumed that free aniline is formed and is then oxidized to phenylhydroxylamine; the phenylhydroxylamine, acting as an oxidizing agent, forms methemoglobin and is itself reduced again to aniline. By this cyclic oxidation and reduction reaction many molecules of methemoglobin can be produced by each molecule of aniline. All of the phenylhydroxylamine is ultimately changed to p-aminophenol, which is excreted.

Heubner, Meier and Rhode (16) and Heubner and Lo-Sing (17) disagreed with the hypothesis of Lipschitz, pointing out that phenylhydroxylamine does not oxidize reduced hemoglobin to methemoglobin and thus cannot participate in the type of cyclic methemoglobin-forming reaction he described. They did, however, concede the possible formation of phenylhydroxylamine, but only as a minor and unimportant metabolite, since they were of the opinion that it is converted rapidly in the blood to azoxybenzene.

Ellinger (13), having isolated a small amount of N-acetyl-phenylhydroxylamine from the blood of cats given acetanilid, and also having found this sub-

stance to be a potent methemoglobin former, postulated still another theory for the metabolism of acetanilid. He assumed that acetanilid is first oxidized to N-acetylphenylhydroxylamine; this is then rearranged to form N-acetyl-o-aminophenol which is oxidized and converted to 2-benzoxazolone which, in turn, is conjugated and excreted in the urine.

Since the earliest investigations concerning the fate of acetanilid in the body it has been repeatedly assumed and implied that the compound forms aniline in the body. Thus Bernheim (18) took the observations of Michel, Bernheim and Bernheim (14) of the hydrolysis of acetanilid *in vitro* by rat tissue as evidence of the formation of aniline in the body after administration of acetanilid. He showed no direct evidence of the occurrence of aniline. The aniline thus formed was assumed to be oxidized to p-aminophenol. He then accepted the hypothesis of Heubner (6) that the p-aminophenol participates in a cyclic reaction with quinone imine, thus producing methemoglobin.

In 1940 Jung (19) agreed with Lipschitz as to the importance of phenylhydroxylamine as a metabolite but disagreed with the assumption that it formed methemoglobin directly. He stated that phenylhydroxylamine *in vitro* is oxidized to nitrosobenzene by oxyhemoglobin. He advanced the concept that the nitrosobenzene formed, oxidizes hemoglobin to methemoglobin and is, in this process, reduced again to phenylhydroxylamine. This reaction places phenylhydroxylamine and its derivative nitrosobenzene as the most important metabolites of aniline. Under Heubner's theory, phenylhydroxylamine, if formed at all, was assumed to produce little methemoglobin and to be quickly transformed to azoxybenzene. Jung pointed out that, as shown by Bamberger (20), azoxybenzene could be formed from phenylhydroxylamine only after condensation with nitrosobenzene, which must first be formed.

The conflicting theories on the metabolism of acetanilid are summarized in figure 1. The metabolites reported as identified in blood or urine are indicated as double-lined squares; those hypothesized, as single-lined squares.

EXPERIMENTAL. *p*-Aminophenol. *Determination of free and conjugated p-aminophenol in urine.* Small quantities of *p*-aminophenol couple with α -naphthol, when heated in an alkaline solution, with the formation of a bright blue pigment. The pigment has a strong light absorption at 620 $m\mu$ and its density is directly proportional to the amount of *p*-aminophenol present.

Four cc. of urine are placed in a test tube and 2 cc. of 6N HCl added. A 2 cc. aliquot of the mixture is then withdrawn for the determination of free *p*-aminophenol and the remainder saved for the determination of total *p*-aminophenol.

This 2 cc. aliquot is placed in a 25 cc. glass stoppered graduate and 7 g. of $K_2HPO_4 \cdot 3H_2O$ added. The mixture is then extracted with 10 cc. of ethylene dichloride by shaking for 10 minutes. The mixture is allowed to stand for a few minutes to clear and a 5 cc. aliquot of the ethylene dichloride extract is withdrawn and reextracted in another 25 cc. cylinder with 15 cc. of 0.1N HCl by shaking for 5 minutes. Ten cc. of the acid extract are placed in a colorimeter tube. A blank is prepared by putting 10 cc. of 0.1N HCl in another colorimeter tube. To each of these tubes are added 2 drops of a 10 per cent solution of resublimed α -naphthol in alcohol followed by 4 drops of concentrated NaOH. The tubes are placed in a water bath maintained at 90°-100°C. for exactly 1 minute and then cooled immediately

in cold water. Readings on the Evelyn colorimeter are made with the blank set at 100 per cent transmission using filter 620 m μ .

The concentration of free p-aminophenol in the original urine, expressed as p-aminophenol hydrochloride, is calculated from the equation:

$$\text{Mg. per cent} = 17.9 L$$

L in this and subsequent equations is the optical density, i.e., 2 minus the log of the per cent transmission. The value 17.9 was obtained from determinations made on solutions of p-aminophenol hydrochloride of known concentrations in urine. The maximum deviation in recoveries was ± 1 per cent. The amount of p-aminophenol found in the 10 cc. aliquot of 0.1N HCl extract used for color development is actually 85.6 per cent of that which would be expected from the diluting and aliquoting of the original sample. This is

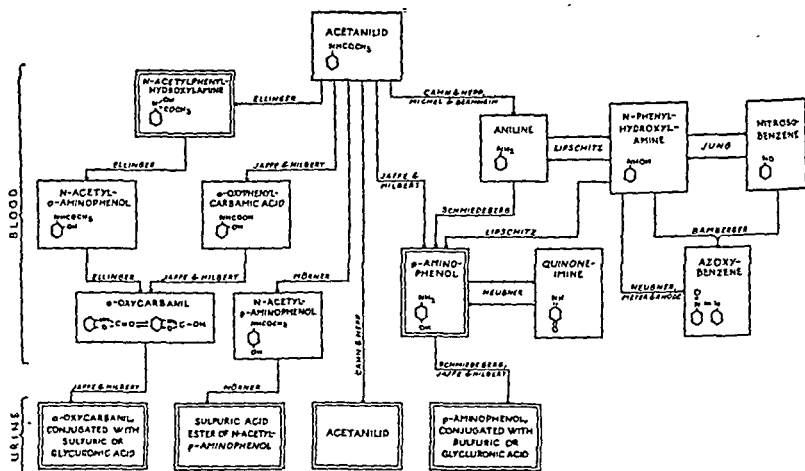


FIG. 1. THE METABOLISM OF ACETANILID ACCORDING TO VARIOUS THEORIES DESCRIBED IN THE LITERATURE

Double lines denote substances that have been reported as identified in blood or urine

probably due to the mutual solubilities of the ethylene dichloride and the aqueous solutions and mainly to the distribution of the p-aminophenol in these media.

It is not desirable to read light transmissions below 30 per cent, corresponding to approximately 9 mg. per cent of p-aminophenol in the urine. Therefore, if this concentration in the urine is exceeded, an adequate dilution of the acid extract is made before coupling with α -naphthol. In analyzing samples of urine the order of magnitude of concentration of p-aminophenol present is often unknown and may easily exceed 9 mg. per cent. It is therefore desirable to carry out the following simple test in each analysis preliminary to the final determination. One cc. of the acid extract is added to 9 cc. of 0.1N HCl in a colorimeter tube and the color developed. From the intensity of color formed the approximate necessary dilution of the acid extract may be judged. This dilution is made with 0.1 N HCl and 10 cc. of the diluted acid extract are used for the final estimation. In calculating the result from the equation given, the figure obtained is multiplied by this dilution.

For the determination of total p-aminophenol the remainder of the original mixture of urine and 6N HCl is hydrolyzed by heating in a glass stoppered tube for 45 minutes in a boiling water bath. Two cc. of the hydrolysate are then treated as for the determination of the free p-aminophenol and the same calculation is used. The result is the concentration

of total p-aminophenol expressed as p-aminophenol hydrochloride. The difference between this and the value obtained for free p-aminophenol represents the conjugated p-aminophenol.

Analysis of normal urine by this method yields a small blank value. The blank values were determined for the hydrolyzed normal urines of 10 subjects and the non-hydrolyzed normal urines of 5 subjects. In the former, the blank values ranged from 0 to 0.59 mg. per cent p-aminophenol hydrochloride, and in the latter, from 0.0 to 0.08 mg. per cent. The average value for each group was respectively 0.32 and 0.02 mg. per cent. These blank values are of negligible significance in studying the urinary excretion of p-aminophenol after ingestion of even subtherapeutic amounts of acetanilid.

Elimination of p-aminophenol and its conjugates. The urinary elimination of p-aminophenol was studied in eight normal male subjects ranging in age from 29 to 46 years. The subjects were given acetanilid in doses of 0.33, 0.65 and 0.98 g. The urine was collected until the rate of excretion of p-aminophenol reached 2 mg. per hour. This rate was found to mark the virtual end of excretion of this substance. Analyses of the total urine of each subject for conjugated and free p-aminophenol were made and the amounts found are expressed here as the hydrochloride. Free p-aminophenol was not detected in the urine of any subject. Three subjects receiving 0.33 g. of acetanilid eliminated 240, 257 and 317 mg. of p-aminophenol in the urine; these values correspond respectively to 69, 74 and 90 per cent of the amount of acetanilid administered. For two subjects receiving 0.65 g. of acetanilid the corresponding values were 604 and 585 mg. and 86 and 84 per cent; and for three subjects receiving 0.98 g., 840, 834 and 866 mg. and 80, 79 and 82 per cent.

These findings confirm the qualitative observations, frequently reported in the literature, that p-aminophenol is a major metabolic end product of acetanilid. They furthermore indicate clearly that in man the p-aminophenol is excreted entirely as a conjugated compound. In all of the subjects the p-aminophenol excreted in the urine represented more than 70 per cent of the acetanilid taken, in the majority 80 to 90 per cent. Considering the likelihood that some of the acetanilid and its products were lost through other avenues of excretion, small margin remains for the urinary excretion of any other major metabolites.

In studying the rate of excretion of conjugated p-aminophenol two subjects were given, in the morning, 0.33 g. of acetanilid, two were given 0.65 g. and two were given 0.98 g. The bladder was emptied by voiding at intervals of 1 to 2 hours during the day, overnight urine was collected, and the bladders again emptied by voiding at frequent intervals for p-aminophenol. The urine obtained at each excretion of p-aminophenol was analyzed separately for p-aminophenol. Figure 2 shows the per cent of the total amount of eliminated p-aminophenol at various periods of time. It will be seen that for the dosages used in these experiments this per cent at any time is approximately the same for all subjects. In 5 hours approximately 50 per cent is excreted; in 10 hours, 80 per cent and in 24 hours, 98 per cent. That the size of the dose has no effect is supported by the fact that the two extreme curves were both obtained with a dose of 0.65 g. of acetanilid while doses of 0.33 g. and 0.98 g. gave curves which fell between these extremes.

The constant relationship between time and per cent of excreted p-aminophenol for various amounts administered, as for any substance excreted from the body whether by kidneys or by lungs, indicates that the rate of excretion is some constant function of the amount of substance present in the body.

It has been suggested in the literature from the observations of Delmas-Marsalet and Estève (21) and Meulengracht and Lundsteen (22) that the

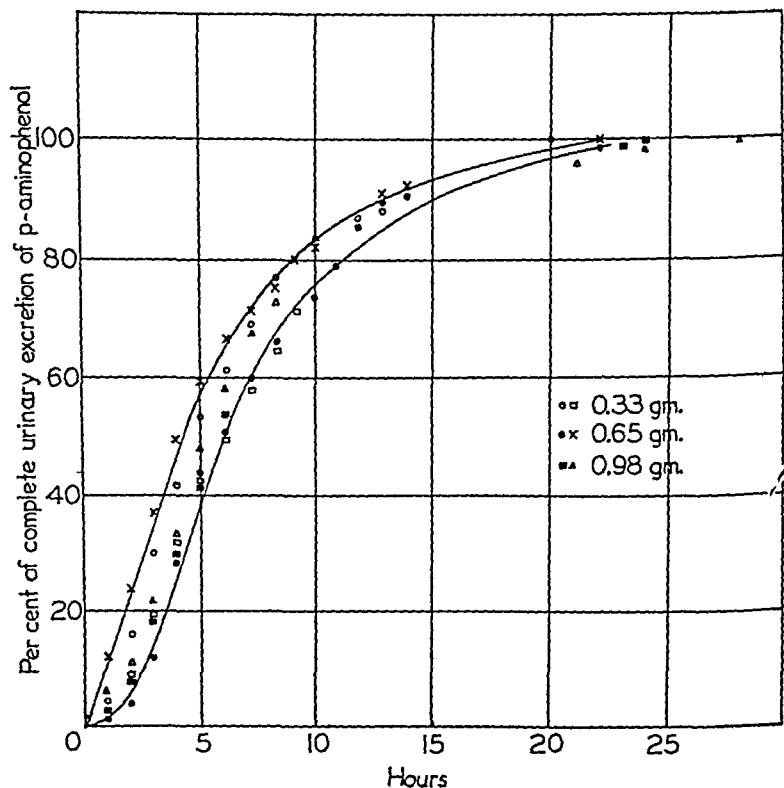


FIG. 2. THE URINARY ELIMINATION OF p-AMINOPHENOL IN PER CENT OF TOTAL EXCRETED AFTER THE ORAL ADMINISTRATION OF 0.33, 0.65 AND 0.98 G. OF ACETANILID TO SIX NORMAL SUBJECTS

accumulation of p-aminophenol in the human body might be responsible for toxic action in chronic acetanilid poisoning with daily doses of this drug as low as 0.49 g. From the data obtained here this seems very unlikely. Even with a daily dose of 0.98 g. of acetanilid any accumulation of p-aminophenol in the body would result in attainment of a state of equilibrium at which the amount of p-aminophenol excreted in 24 hours would be equal to that produced by the acetanilid. Since in 24 hours 98 per cent of the p-aminophenol is excreted, the

maximum amount, x , present in the body at equilibrium after each daily dose of 0.98 g. may be calculated from the equation

$$98\% x = 0.98 \text{ g.}$$

The value thus obtained is 0.99 g. Since, under these conditions, 0.98 g. per day is eliminated, a maximum accumulation at any time of 0.01 g. may be anticipated, an amount which is negligible.

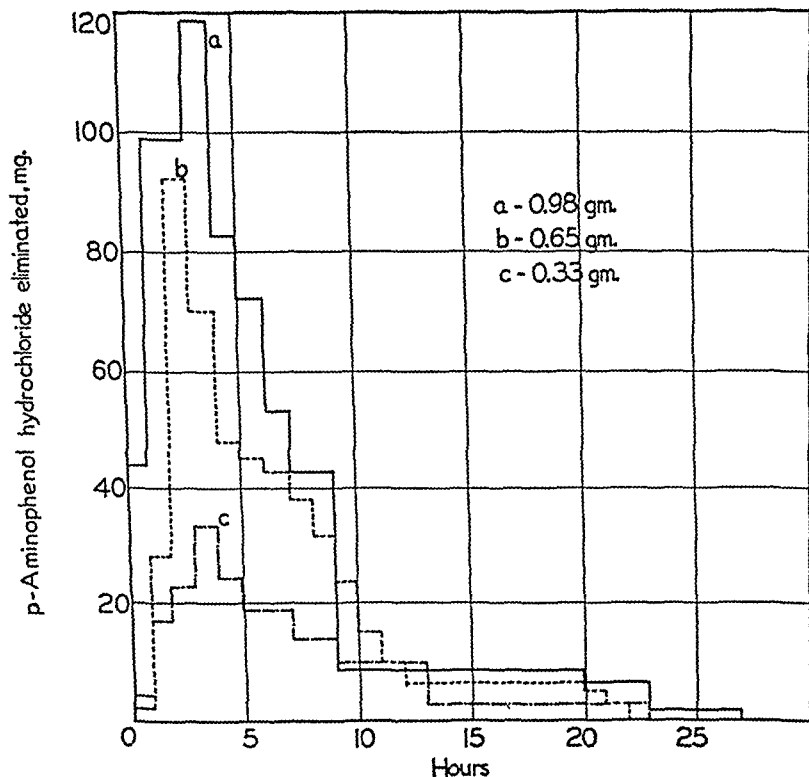


FIG. 3. THE HOURLY ELIMINATION OF p-AMINOPHENOL IN THREE NORMAL SUBJECTS AFTER ORAL ADMINISTRATION OF 0.33, 0.65 AND 0.98 G. OF ACETANILID

Figure 3 shows the hourly elimination of p-aminophenol in mg. after doses of 0.33, 0.65 and 0.98 g. It is seen that the elimination of p-aminophenol starts within a short time after administration of the acetanilid, irrespective of the dose, and during the third and fourth hours reaches a maximum amount. Thereafter the amount diminishes. Such a pattern for the elimination of p-aminophenol would be expected from the curves of Figure 2 and can be explained only by a rapid transformation of acetanilid in the body to p-aminophenol.

p-Aminophenol is capable of conjugation on the amino group with the acetyl radical and on the hydroxyl group with sulfuric or glycuronic acid. All of these conjugates have been detected in the urine after administration of acetanilid, but no data have been reported as to the proportions of the different conjugates. Such data are significant because of possibly different pharmacological action of these conjugates.

In the present work, quantitative determinations were made of the amounts of amino and hydroxyl conjugates of p-aminophenol excreted in the urine in man after administration of acetanilid. N-acetyl-p-aminophenol is only slightly soluble in water; and, moreover, the hydroxyl group does not form a salt with weakly alkaline dibasic potassium phosphate. The hydroxyl conjugates are more soluble in water, and, furthermore, readily form salts with the potassium phosphate. These salts are extremely soluble in water and are not extracted by ethylene dichloride. For the separate determination of N-acetyl-p-aminophenol and the hydroxyl conjugates in urine, one portion of urine was hydrolyzed and analyzed in the usual manner for total p-aminophenol. Another portion was made up to 10 cc. with water in a 100 cc. glass stoppered cylinder, 40 g. of dibasic potassium phosphate were added, and the mixture extracted with 25 cc. of ethylene dichloride by shaking for 10 minutes. A 20 cc. aliquot of the ethylene dichloride extract was then re-extracted with 7 cc. of 0.1N NaOH. A direct estimation of the N-acetyl-p-aminophenol in this alkaline extract was then made by hydrolyzing an appropriate aliquot made 2N in HCl, developing the color with α -naphthol and concentrated NaOH and reading with the Evelyn colorimeter. Calibration of the colorimeter for this estimation was carried out by determining the photometric density of known amounts of N-acetyl-p-aminophenol in 0.1N NaOH treated in the same manner as the alkaline extract described above. Repeated analyses of normal urine samples containing known amounts of N-acetyl-p-aminophenol have shown that in the extractions used here, with allowance for aliquoting, 89 per cent of the N-acetyl-p-aminophenol is extracted with maximum deviation of ± 2 per cent. From the amount of N-acetyl-p-aminophenol found in the alkaline extract, the concentration in the original urine may thus be calculated. The difference between the latter value and that obtained for total p-aminophenol of the urine represents the concentration of hydroxyl conjugated p-aminophenol present since, as shown, there is no free p-aminophenol present.

In a subject receiving 0.98 g. of acetanilid, the urine was collected for 24 hours and analyzed for total p-aminophenol and N-acetyl-p-aminophenol. The urines excreted during the first, second and ninth hours after administration of the acetanilid were collected separately and analyzed separately for total p-aminophenol and N-acetyl-p-aminophenol. In the entire 24 hour sample the amount of N-acetyl-p-aminophenol was 4.6 per cent of the total p-aminophenol excreted. In the first hour sample, however, it was 17.9 per cent; in the second hour, 19.6 per cent; and in the ninth hour, only 4.1 per cent. The fact that the proportion of N-acetyl-p-aminophenol excreted during the first few hours after administration of acetanilid is high, strongly suggests that the greatest concentration of this conjugate exists in the blood at the time when the pharmacological effects of acetanilid are most pronounced.

ANILINE. *Determination of free and conjugated aniline in urine.* Small amounts of aniline may be accurately determined by the color reaction occurring when the aniline is diazotized and coupled with α -naphthol. The intensity of the color is a linear function of the amount of aniline present and shows a strong light absorption at 490 $m\mu$. The reaction

is not only sensitive but it is also specific; none of the known derivatives of aniline, in concentrations ever found in urine, causes interference.

Four cc. of urine are placed in a test tube and 2 cc. of 6N HCl added. A 2 cc. aliquot of the mixture is then withdrawn for the determination of free aniline and the remainder saved for the determination of conjugated aniline.

This 2 cc. aliquot is placed in a 25 cc. glass stoppered cylinder and made alkaline with 8 drops of concentrated NaOH. The solution is then extracted with 10 cc. of ethylene dichloride by shaking for 5 minutes. The mixture is allowed to stand for a few minutes to clear and a 5 cc. aliquot of the ethylene dichloride extract is reextracted in a 25 cc. glass stoppered cylinder with 15 cc. of 0.1N HCl by shaking for 5 minutes. Ten cc. of the 0.1N HCl extract are pipetted into an Evelyn colorimeter tube. A blank for comparison is prepared by placing 10 cc. of 0.1 HCl in a second colorimeter tube. To the contents of each tube 2 drops of a 10 per cent solution of sodium nitrite are added and, after mixing, 4 drops of a saturated solution of ammonium sulfamate. When the evolution of nitrogen ceases, 2 drops of a 10 per cent solution of resublimed α -naphthol in alcohol are added and, after mixing, 4 drops of concentrated NaOH are added. A reading is then taken on the Evelyn colorimeter with the blank set at 100 per cent transmission using filter 490 m μ . The concentration of free aniline as aniline hydrochloride in the original urine is calculated from the equation:

$$\text{Mg. per cent} = 6.97 L$$

The value 6.97 was derived by analyzing, as described, urine containing known concentrations of aniline. The maximum deviations in recoveries were ± 2 per cent. The amount of aniline found in the 10 cc. aliquot of 0.1N HCl extract used for final color development is actually 103 per cent of that anticipated from the various dilutions. The deviation is probably due to the mutual solubility of the ethylene dichloride and the aqueous solutions. The sensitivity of the method is such that the maximum concentration of aniline in the urine, which can satisfactorily be read on the colorimeter, is approximately 3 mg. per cent. For higher concentrations it is necessary to dilute the urine.

For the determination of total aniline the remainder of the original mixture of acidified urine is hydrolyzed by heating in a stoppered test tube for 45 minutes in a boiling water bath. Two cc. of the hydrolysate are then pipetted into a 25 cc. glass stoppered cylinder and treated as for the determination of free aniline described above. The same calculation is used, the result being the concentration of total aniline. The concentration of conjugated aniline as hydrochloride is taken as the difference between free and total aniline.

Normal urine, subjected to the analytical procedure described, yields a small blank value. The values were determined for the non-hydrolyzed and hydrolyzed urines of five individuals. The non-hydrolyzed urines gave values of 0.024 to 0.071 mg. per cent with an average of 0.041. In the hydrolyzed urines they varied from 0.024 to 0.063 mg. per cent with an average of 0.041. These values are insignificant in studying the urinary excretion of aniline after the ingestion of even small quantities of acetanilid.

Elimination of aniline and acetanilid. It has been suggested in the literature that part of the acetanilid ingested is eliminated as such by the kidneys (2) and also that aniline is formed in the body by the hydrolysis of acetanilid (18). The urinary excretion of free and conjugated aniline after administering acetanilid in doses of 0.33, 0.65 and 0.98 g. was determined in five subjects. In no instance was any free aniline found at any time in the urine. Minute quantities, from 1.12 to 2.43 mg. of conjugated aniline were excreted by all of the subjects; these amounts correspond to 0.2 to 0.6 per cent of the amounts of acetanilid given. The excretion of the conjugated aniline occurred mainly during the first 4 hours after taking the acetanilid and ceased many hours before that of p-aminophenol.

2-Benzoxazolone. The occurrence of large amounts of 2-benzoxazolone in the urine of dogs after giving acetanilid, as reported by Jaffe and Hilbert (4), suggested to them that this substance is a major metabolite in these animals. This suggestion seems to be further supported by Ellinger (13) who identified N-acetylphenylhydroxylamine in the blood of cats receiving acetanilid and concluded that this material is transformed to 2-benzoxazolone. The possibility is therefore suggested that in man the part of the acetanilid which is not accounted for by the urinary excretion of p-aminophenol may be excreted as 2-benzoxazolone. This possibility was tested.

One subject was given 0.98 g. of acetanilid and the urine collected for 24 hours. The urine was made 2N in HCl and hydrolyzed by boiling for 30 minutes. The hydrolysate was extracted three times with 400 cc. portions of ethylene dichloride. The combined extract was washed once with 100 cc. of water, twice with 100 cc. portions of a 2 per cent solution of sodium bicarbonate and again with 100 cc. of water. The ethylene dichloride was then dried with Na_2SO_4 and distilled. The residue was sublimed at 120°C . under reduced pressure (1 mm. Hg). The sublimate consisted only of a slight amount of a brown oily material which, when treated to form benzoxazolone benzoate by reaction with benzoylchloride in pyridine yielded no crystals of the benzoate.

Similar treatment of a normal 24 hour urine specimen gave the same result. If, however, as little as 10 mg. of 2-benzoxazolone are added to a volume of normal urine, corresponding to that eliminated in 24 hours, the sublimate consists of a noticeable deposit of amorphous benzoxazolone which, by the treatment with benzoylchloride in pyridine, yields, on crystallization, sufficient benzoxazolone benzoate for identification by melting point and mixed melting point. It was concluded from these experiments that in man, after acetanilid, no detectable quantity of 2-benzoxazolone is excreted.

Azo compounds. Jung (19) postulated phenylhydroxylamine and nitrosobenzene as intermediary metabolites of acetanilid. As pointed out by Bamberger (20) these two compounds readily combine to form azoxybenzene. The body may transform this into other azo compounds. The method of analysis used here for azoxy, azo and hydrazo-benzene is based on the fact that they are readily reduced with zinc, each molecule yielding 2 molecules of aniline. One subject was given 0.98 g. of acetanilid and the urine was collected for 24 hours. Four cc. of the urine, together with 2 cc. of 6N HCl, were hydrolyzed by heating. Analysis of this hydrolysate for aniline hydrochloride showed 1.32 mg. of this substance in the 24-hour urine. Such a small amount of aniline would not interfere with the determination of any appreciable quantity of aniline that might be formed by the reduction of azo compounds. Another 4 cc. portion of the 24-hour urine sample was added to 2 cc. of 6N HCl and hydrolyzed. Powdered zinc was then added for reduction. The reduced hydrolysate was then analyzed for aniline. From the additional aniline formed by reduction it was calculated that 4.13 mg. of an azo compound as azoxybenzene were eliminated in 24 hours. This represents approximately 0.5 per cent of the acetanilid given.

The solubility of the azo compounds in water is extremely low. Their occur-

rence in the urine, in amounts greater than those found, would not be expected. However, they could be oxidized to hydroxylated compounds in the body, thus making them more soluble and hence more readily excreted in the urine. Analysis of the urine for p-hydroxy compounds of azo, azoxy and hydrazobenzene is based on the fact that they are readily reduced with zinc, each molecule of monohydroxy compound yielding 1 molecule each of aniline and p-aminophenol, and each molecule of dihydroxy compound yielding 2 molecules of p-aminophenol. A subject was given 0.65 g. of acetanilid and the urine collected for 24 hours. To this urine (approximately 1,000 cc.) was added 200 cc. concentrated HCl and the mixture was hydrolyzed by refluxing for 3 minutes. The hydrolyzed urine was then extracted with three 150 cc. portions of CCl_4 . The combined extract was then washed three times with 25 cc. portions of 0.1N HCl to remove traces of aniline and p-aminophenol; it was then washed three times with 25 cc. portions of water. The CCl_4 was then extracted with two 10 cc. portions of 0.2 N NaOH. The combined alkaline aqueous extract was then made acid with 5 cc. of 6N HCl, reduced by the addition of powdered zinc and taken down with heat to a volume of 5 cc. One 2 cc. portion of this was then analyzed for aniline and another 2 cc. portion for p-aminophenol. No aniline was found, indicating absence of any monohydroxy azo compound. A small amount of p-aminophenol was found, corresponding to approximately 40 micrograms of p-dihydroxy azo compound excreted in 24 hours. A similar analysis was made of a 24-hour urine sample in another subject after 0.98 g. of acetanilid. No monohydroxy azo compound was excreted but approximately 45 micrograms of p-dihydroxy azo compound was excreted.

SUMMARY

1. The present theories of the metabolism of acetanilid in man are conflicting. As the first step toward clarification a quantitative study was made of the major metabolites appearing in the urine.
2. Seventy to 90 per cent of the acetanilid administered appeared in the urine as conjugated p-aminophenol.
3. There was no free p-aminophenol.
4. Of the total p-aminophenol excreted, as determined in one experiment, 4 per cent consisted of the N-acetyl conjugate and 96 per cent as an hydroxy conjugate. The major portion of N-acetyl-p-aminophenol was excreted in the first few hours after administration.
5. Conjugated aniline was excreted to the extent of 0.2 to 0.6 per cent of the amount of acetanilid administered and mainly during the first 4 hours.
6. There was no free aniline.
7. There was no 2-benzoxazalone.
8. Azo compounds appeared in the urine in an amount corresponding to about 0.5 per cent of the acetanilid administered.

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ARSINE POISONING, MODE OF ACTION AND TREATMENT¹

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Arsine (AsH_3) or arseniuretted hydrogen has been the subject of numerous investigations because of the number of fatal poisonings attributed to this agent (1). The two most characteristic features of arsine poisoning are the marked intravascular lysis of erythrocytes and renal damage. The extent of hemolysis and renal damage is roughly proportional to the exposure to arsine (2). In massive doses of arsine, blood destruction is almost complete and renal damage is of little importance. However, in exposures close to the L.D.100, animals usually survive 24-48 hours and show marked renal damage as well as extensive (80-90%) intravascular hemolysis.

The purpose of this report is to present a brief summary of the experiments which have led to the finding of an effective therapeutic agent for the treatment of arsine poisoning, exposures ca. L.D.100, providing the treatment is initiated within a short time after exposure. In this study advantage was taken of the earlier observations that arsine will hemolyze erythrocytes *in vitro* in the presence of oxygen. It was assumed that if compounds could be obtained which would prevent the hemolysis of the erythrocytes *in vitro* that one of these might also be effective *in vivo*. As arsine and arsenious acid, an oxidation product of arsine in contact with hemoglobin (3), also inhibit the respiration of tissue slices, including kidney, the ability of these compounds to protect tissue respiration *in vitro* was also determined.

The progress made in this investigation was largely dependent on the success of the British workers leading to the discovery (4) that a dithiol, 2,3-dimercaptopropanol, is effective in the treatment of lewisite (β -chlorvinylchlorarsine) poisoning. Although this compound has not proven to be of much value in the treatment of arsine poisoning a closely related compound, 2,3-dimercaptopropyl ethyl ether, has done so.

METHODS. In all cases the arsine was generated from magnesium arsenide and water immediately before use. Arsine-oxygen mixtures were prepared by mixing known volumes of gas and the concentration of the arsine was checked chemically using a modification of the molybdenum blue reduction technique (5).

The lysis of erythrocytes by arsine *in vitro* was determined in the following manner: 1.6 ml. of defibrinated human blood plus 0.4 ml. of serum (with or without compounds being tested for protective effect) were exposed to a 100 ml. gas phase consisting of 0.20% arsine-99.80% oxygen in closed, rotating tonometers and incubated at 37.5°C. for two hours. When compounds were added to test their protective effect they were introduced into the system five (5) minutes after the cells were exposed to arsine. The per cent lysis was determined in a photoelectric colorimeter by measuring the relative amounts of hemoglobin in solution and the total hemoglobin present.

¹ The work described in this paper was done under a contract recommended by the Committee for Medical Research between the O.S.R.D. and Memorial Hospital, 1942-1943.

The inhibition of tissue respiration by arsine *in vitro* and its prevention by the addition of suitable compounds was determined in the following manner: 150 mg. wet weight of tissue slices were suspended in a media consisting of 1 part Krebs-Ringer-Phosphate-Glucose solution and one part neutralized horse serum (6) and their respiration measured in standard Warburg vessels. The protective compounds were added and then the arsine-oxygen mixtures were flushed through the manometers. Under the conditions of these experiments the respiration of the control tissues held up well for three hours.

TABLE 1

Prevention of the lysis of human erythrocytes caused by arsine in vitro

COMPOUND	PER CENT LYSIS
Control—no compound added	33.3
2,3-dimercaptopropyl ethyl ether	4.8
1,2-dimercaptopropane	4.9
2,3-dimercaptopropyl methyl ether	5.0
2,3-dimercaptobutane	5.1
ethylmercaptoethylsulfide	5.1
methyl glucamine formaldehyde-2,3 dimercapto propyl ethyl ether	5.3
sec octyl mercaptan	5.4
2,3-dimercaptopropyl butyl ether	5.9
N-(2,3-dimercaptopropyl) carbamate	5.9
lauryl mercaptan	6.0
n-amyl mercaptan	6.8
methyl glucamine formaldehyde-1,3-dimercaptopropane	7.0
1,2-dimercaptoethane	7.0
2,3-dimercaptopropyl acetate	7.2
cyclohexyl mercaptan	7.2
1,6-dimercaptohexane	7.9
methyl glucamine formaldehyde-1,2,3-trimercaptopropane	8.2
2,3-dimercaptopropyl chloride	8.3
n-butyl mercaptan	8.4
1,3-dimercaptopropane.	8.8

Arsine concentrations equal 0.20% in 100 ml gas phase

Protective compounds 1×10^{-2} M were added five minutes after cells were exposed to arsine. Per cent lysis was determined at the end of a two hour incubation period at 37.5°C.

The animals were gassed in a chamber in which the total volume was 494 liters. Chemical determination of the arsine concentrations showed that in the absence of animals the arsine concentration did not decrease significantly in two hours. Rabbits were exposed in groups of three and monkeys and dogs individually.

RESULTS. Of the 150 compounds which have been tested, mono and dithiols have been found to give the best protection against the lysis of erythrocytes by arsine *in vitro*. Ascorbic acid and sodium thiosulfate were found to be effective but not in equivalent concentrations.

A list of the most effective compounds in order of decreasing effectiveness is given in table 1. All of these agents were added five minutes after the erythrocytes (whole blood) were exposed to a standard amount (100 ml.) of 0.20% arsine. A marked and consistent difference in the sensitivity of different individ-

ual's erythrocytes to lysis by arsine was noted: i.e., 40 to 48% lysis of one donor's occurred consistently upon exposure to the standard amount of arsine whereas only 22 to 28% lysis of another donor's blood was obtained. The average percent of lysis for over 150 experiments on blood from 25 individuals was 33.3%. The amount of protection afforded can be seen in table 1 as the difference between the amount of hemolysis in the control and the lysis in the presence of the compound being tested. For the purpose of comparing different experiments, the amount of lysis in the control was corrected to 33.3% and the lysis in the protected vessels corrected proportionately. The percent lysis figures for each of the compounds listed are based on from 5 to 12 experiments. 2,3-dimercaptopropyl ethyl ether is one of the best, if not the best, of all the compounds tested. BAL(2,3-dimercaptopropanol) which is so effective against lewisite is much less effective than any of the other compounds listed: i.e., 17% lysis. The long chain aliphatic monothiols that were tested were surprisingly effective: i.e., sec. octyl mercaptan 5.4%, lauryl mercaptan 6.0%. Good protection could also be obtained by using high concentrations of ascorbic acid or sodium thiosulfate although they are only about 10% as effective as the thiols at 1×10^{-3} M concentration.

Of the compounds which have been found to give good protection against the lysis of erythrocytes by arsine only one group, the dithiols, was found to give good protection *in vitro* to tissue slice respiration. Monothiols, ascorbic acid, and sodium thiosulfate did not significantly protect in four times the concentration of dithiols. As in the case of erythrocyte lysis, different dithiols afforded varying degrees of protection. A comparison of the effectiveness of the dithiols was made using kidney, brain and liver tissue. 2,3-dimercaptopropyl ethyl ether, 1,3-dimercaptopropanol and 1,3-dimercaptopropane afforded almost complete protection of kidney and brain tissue and some protection of liver slice respiration. 2,3-dimercaptopropanol (BAL) gave good protection of kidney and brain but accelerated the inhibition of liver slice respiration by arsine. A typical experiment is shown in figure 1. In the concentrations used 2,3-dimercaptopropyl ethyl ether by itself had no effect on kidney slice respiration.

In view of the encouraging results obtained with the mono and dithiols in the *in vitro* tests attempts were then made to treat arsine poisoning *in vivo* with these agents. As BAL was the first dithiol available it was tested first. Some protection against intravascular lysis was obtained with BAL in rabbits but even with the most effective dosage schedule the results were erratic. Our results with BAL are in agreement with those obtained by Lovatt-Evans (7) who characterized BAL as at best "a desperate remedy" for arsine poisoning. It was noted in our experiments that whereas the L.D.50 dose of BAL is ca. 100 mg./kg. in normal rabbits, this drops to ca. 30 mg./kg. in arsine poisoned rabbits. These results are in marked contrast to those obtained with lewisite where several lethal doses of BAL will completely neutralize several lethal doses of lewisite.

As the results of the *in vitro* tests indicated that there were a number of compounds which were much better in preventing the lysis of erythrocytes by arsine

than BAL several of these agents were also tested *in vivo*. The data obtained on several of these compounds which were studied most extensively are shown in

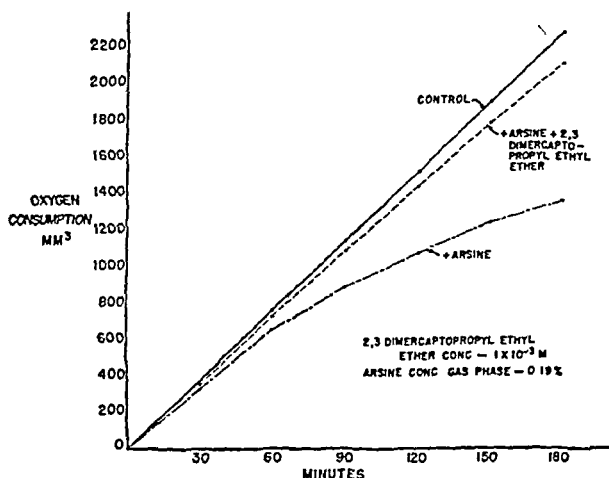


FIG. 1. THE PROTECTION OF KIDNEY TISSUE SLICE RESPIRATION FROM INHIBITION BY ARSINE

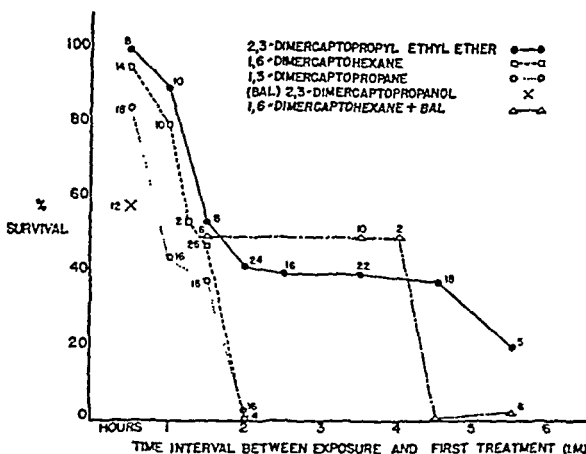


FIG. 2. DELAYED TREATMENT OF ARSINE POISONING IN RABBITS

figure 2. Only those rabbits treated at the most successful dosage schedule are included. In these experiments the pure compounds, no solvents, were injected

intramuscularly in the following amount:

	1ST INJ.	2ND INJ.	3RD INJ.
	mg./kg.	mg./kg.	mg./kg.
2,3-dimercaptopropyl ethyl ether.....	30	15	5
1,3-dimercaptopropane.....	25	20	10
1,6-dimercaptohexane.....	250	100	25
BAL.....	25	20	10
BAL & 1,6-dimercaptohexane.....	10-250	0-100	0-100

In figure 2 the time interval between the gassing and treatment refers to the time period between the beginning of the exposure and the time at which the first injection was given. The second injection was given four hours after the first and the third injection twenty-four hours later. The rabbits were exposed to a concentration of 0.45 mg. of arsine per liter for thirty minutes in groups of three. One rabbit from each group was kept as the control and the other two were treated. Of the 140 controls 125 died. As is indicated in figure 2 the ethyl ether of 2,3-dimercaptopropanol was found to be the most effective single compound administered. 1,3-dimercaptopropane and 1,6-dimercaptohexane were less effective. The combination of 1,6-dimercaptohexane and BAL was more effective than either alone.

Cattell and his associates (8) found that the ethyl ether of 2,3-dimercaptopropanol if injected intravenously in cats would produce permanent damage to the central nervous system. The use of this compound in the treatment of arsine poisoning in our laboratory in the monkey, dog and rabbit by the intramuscular route was accomplished without any evidence of central nervous system damage. Cattell and his associates were not able to produce this c.n.s. damage in the monkey or the dog. However, as Cattell did not observe c.n.s. damage in the cat when the compound was applied percutaneously the use of this route of application in the treatment of arsine poisoning was investigated.

It was found that the percutaneous administration of this compound was an effective way to treat arsine poisoning in the rabbit, dog and monkey. Experiments carried out in collaboration with Riker and Cattell showed that cats could also be treated successfully without any evidence of systemic toxicity due to the compound appearing. The data showing the results of the treatment of arsine poisoning by the percutaneous administration of 2,3-dimercaptopropyl ethyl ether are summarized in table 2.

The course of the poisoning in three typical monkeys exposed to arsine (L.D. 80), 0.45 mg./liter for 15 minutes, is shown in figure 3. Monkey A died in 24 hours showing marked intravascular hemolysis and hematuria. Monkey B presents a typical picture of the occasional animal (monkey, dog or rabbit) which survived without any treatment. The erythrocyte count dropped 65% in 24 hours reaching a minimum value by the 3rd to 4th day. The blood urea N

rose to a maximum on the 6th day. The number of reticulocytes rose to 20% of the red blood cells on the 3rd day. NPN blood levels paralleled the urea N. Blood lactic acid concentration also increased, 19 mg.% to 100 mg.%. This

TABLE 2

Results of the treatment of arsine poisoning by the percutaneous application of 2,3-dimercaptopropyl ethyl ether

DOSE mg./kg.	SPECIES	TIME INTERVAL BETWEEN EXPOSURE AND APPLICATION HOURS	RESULTS	
			No. surv.	No. dead
0	Rabbit		4	19
75	Rabbit	0.5	10	1
75		3.5		
25		24		
65	Rabbit	1	6	0
65		4		
25		24		
65	Rabbit	2.5	2	2
65		5.5		
25		24		
65	Rabbit	3	1	1
65		6		
25		24		
0	Monkey		1	4
100	Monkey	0.5	2	0
100		4.5		
50		24		
0	Dog		0	5
40-60	Dog	0.5	5	0
40-60		4.5		
25		24		
60	Dog	2	4	0
60		6		
60		24		

monkey could scarcely raise himself from the floor of his cage from the 2nd to the 7th days. The only food this monkey would consume was oranges. Monkey C was given the same exposure to arsine as monkeys A and B but was treated by the percutaneous application of 100 mg./kg. of 2,3-dimercaptopropyl ethyl ether

0.5 and 4.5 hours after exposure and 50 mg./kg. 24 hours later. As the data presented in figure 3 indicate, the speed of the lysis of erythrocytes was greatly reduced and normal renal function (blood urea N) was maintained. As can be seen in this figure the ultimate lysis of erythrocytes is not prevented. The red cell count usually reaches a minimum on the 6th or 7th day after exposure. The continued administration of 2, 3-dimercaptopropyl ethyl ether on the third and successive days has not prevented this slow lysis of erythrocytes in any of the animals tested. In marked contrast to monkey B, monkey C and other treated

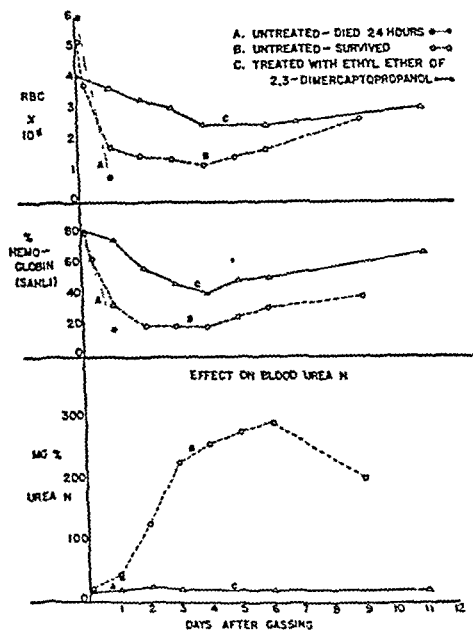
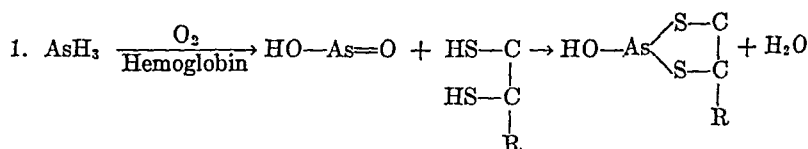


FIG. 3. EFFECT OF ARSINE POISONING IN MONKEYS

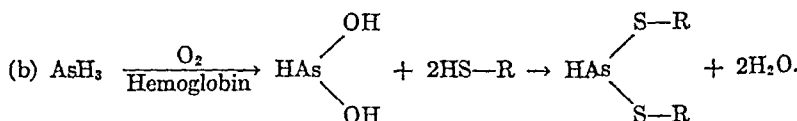
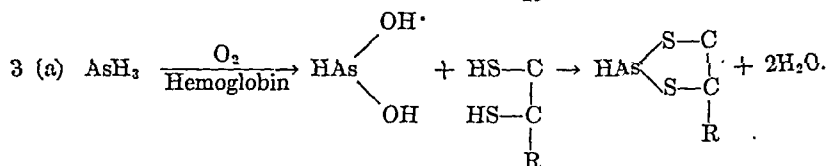
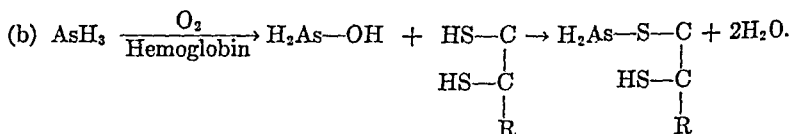
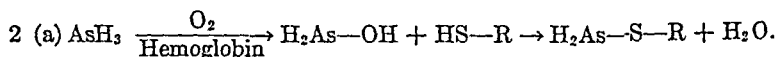
animals at no time during the course of recovery appeared to be very ill. Food consumption was almost normal.

DISCUSSION. One of the interesting features of the protection of arsine lysis of erythrocytes *in vitro* is the observation that monothiol of the aliphatic type are approximately as effective as the best dithiols. Due to our lack of knowledge of the mechanism of lysis of erythrocytes by arsine it is not possible to offer any simple explanation of this observation. Arsine is known to be lytic only under aerobic conditions but both of the stable oxidation products, arsenic trioxide and arsenic pentoxide, are not lytic. Hence it is probable that an intermediary oxidation product is the lytic agent. Arsine has been found not to react with dithiols in aqueous solution (7), however, arsenic oxides, arsenites, and arsenates

will react. As the stable arsenic oxides are not lytic the following reaction probably plays no role in the prevention of lysis:



However reactions of intermediary oxidation products of arsine with thiols may occur:



Under the conditions of our experiments the molecular ratio of arsine (lytically effective) to thiol is approximately one to one. The molecular ratio was higher (1.5-1) than this but small amounts of arsine can be introduced into the system without causing any erythrocytolysis within the two hour test period. In order to gain a better understanding of this aspect of the problem, chemical isolation experiments are needed to determine the type of arsenic-thiol compound formed.

The data obtained in these experiments indicates that the reason for the effectiveness of the thiol therapy *in vivo* in protecting renal function lies, to a large extent, in the prevention of erythrocyte lysis. . . for example, BAL gives excellent protection to kidney slice respiration *in vitro* when exposed to arsine but is not very effective in preventing lysis of erythrocytes. This compound has not been found to be very good for treatment in animals. Also, a monothiol, sec. octyl mercaptan, which affords excellent protection against lysis of erythrocytes *in vitro* and no protection of kidney slice respiration *in vitro*, has *in vivo* (six rabbits) been found to slow down the rate of intravascular hemolysis and saved the animals.

Harrison, Ordway and Albrink (9) in an extension of these experiments on arsine have found that the ethyl ether of 2, 3-dimercaptopropanol was effective in the treatment of arsine poisoning in dogs, whereas BAL was not.

In this report it is not intended to imply that 2, 3-dimercaptopropyl ethyl ether is the best therapeutic agent of all the compounds listed in table I. This compound is the best of the few that have been examined adequately *in vivo*. One other compound, 1, 2-dimercaptoethane has been found by Levvy (10) to be effective in the treatment of arsine poisoning in mice. No tests have been conducted in this laboratory using mice although 2, 3-dimercaptopropyl ethyl ether has been found to be effective in treating arsine poisoning in rats.

The application of 2, 3-dimercaptopropyl ethyl ether to the skin of twelve volunteers has not given any evidence of irritation. Gilpin, et al. have applied percutaneous doses of 50 mg./kg. to human subjects without any observable ill effects (11).

SUMMARY

1. "Fat soluble" mono and dithiols have been found to prevent the lysis of erythrocytes by arsine *in vitro* when they are added five minutes *after* the cells have been exposed to arsine.

2. Certain dithiols will protect tissue respiration from inhibition by arsine and arsenite.

3. Several dithiols are effective in saving the lives of rabbits, dogs and monkeys given doses of arsine ca. the L.D.100.

4. 2,3-dimercaptopropyl ethyl ether has been shown to be effective in the treatment of arsine poisoning in rabbits, monkeys and dogs when administered either intramuscularly or percutaneously.

5. The effectiveness of this compound in the treatment of arsine poisoning falls off rapidly as the time interval between exposure and treatment is increased.

6. Because of the possible danger of central nervous system damage caused by the injection of this compound into a vein, the percutaneous route of administration is to be preferred.

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THE EFFECT OF CARBON TETRACHLORIDE INDUCED LIVER INJURY UPON THE ACETYLCHOLINE HYDROLYZING ACTIVITY OF BLOOD PLASMA OF THE RAT¹

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The blood plasma of rat, dog, man and other mammalian species contains one (or possibly several related) proteins which are capable of catalyzing the hydrolysis of certain choline esters as well as of simple esters. The enzymatic activity of these proteins permits their determination not only in blood plasma but also in tissue homogenates.

The present paper deals with the effect of carbon tetrachloride poisoning upon the plasma cholinesterase activity of the rat. It represents a first attempt to experimentally define the site of origin of this protein, and to search for factors which might interfere with a straightforward interpretation of the plasma cholinesterase values determined.

METHODS. The variables studied included body weights, liver and plasma cholinesterase activities, erythrocyte counts, "liver function," organ weights, and pathological histology of livers and kidneys.

All experiments were performed on female albino rats kept on a stock diet of Purina Fox Chow in the laboratory during and for at least one week prior to each experiment.

Five to ten experimental rats and as many control animals were employed for each individual experiment. In view of the marked influence of age upon the plasma cholinesterase activity of young female rats, in each experiment animals weighing within $\pm 20\%$ of the mean body weight of the groups were chosen; the initial mean weights of control and experimental groups were not allowed to differ by more than 5%. The range of mean weights covered by all of the groups together was from 110 to 210 grams. During the experiments all animals were weighed on alternate days.

Liver injury was produced by the intraperitoneal administration of carbon tetrachloride (C.P. grade). A narrow range (0.5–0.85 cc. per kg.) of carbon tetrachloride doses was explored for the purpose of finding a dose which would produce liver damage in the majority of the animals with minimal mortality and minimal extrahepatic damage. The dose which appears to satisfy these requirements is 0.5 cc. per kg. every second day. This procedure has been found to lead to a fairly reproducible degree of liver injury which can be maintained at least until the tenth day of the experiment, with only minor extrahepatic manifestations. The discussion of results will be based upon the data obtained with this dose, and deviations seen with the higher dose levels will be mentioned where necessary.

Blood samples for determinations of plasma cholinesterase activity were obtained by heart puncture. 2.0 mgm. of mixed oxalates (1) were added as anticoagulant to each cc. of blood. In each case at least twelve hours elapsed between the last carbon tetrachloride injection and the time of bleeding. The determinations were carried out according to Ammon's manometric procedure (2), using 0.03 M acetylcholine bromide (Hoffman-La Roche, once recrystallized) in physiological saline-bicarbonate buffer (0.025 M) at pH 7.4 and 37.8°C. Results are expressed in millimols of acetylcholine hydrolyzed per liter of

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plasma per hour. The same procedure was applied to the determination of the cholinesterase activity of liver homogenates prepared by homogenizing about one-half gram of freshly obtained liver with 10 cc. of buffer at 0°C. in a Potter-Elvehjem homogenizer. The uncentrifuged homogenates were employed; the results are given in millimols of acetylcholine hydrolyzed by 1000 gm. of fresh tissue per hour.

Erythrocyte counts were obtained on blood from the tip of the tail of the rats.

Prothrombin times were determined by the method of Kato and Poncher (3). Within the rather wide limits of variation of data obtained by this method, no significant differences between normal and carbon tetrachloride treated animals could be observed.

A newly developed procedure was employed to provide an index of the degree of liver damage produced without requiring extensive manipulation or bleeding of the rats. This test is based upon the detoxication of certain barbiturates by the liver and the impairment of this function in certain forms of toxic hepatitis (4). The test is carried out by the intraperitoneal administration of 1% Sodium Evipal in physiological saline in doses of 50 mg. per kg. of rat. The period of anesthesia is determined as the period during which a rat will retain side position, even if stimulated by dropping a small weight on the animal's tail. Following toxic liver injury by carbon tetrachloride the duration of anesthesia is prolonged several-fold. (See figure 2.)

Within 60 minutes after bleeding all animals were sacrificed by a blow on the head. At autopsy the weights of the livers, and in some experiments also of kidneys and of uteri were determined. Specimens for pathological examination were fixed immediately in Zenker's solution and stained with hematoxylin and eosin.

RESULTS. On the diet employed the normal rats showed a growth rate of about 3 grams per day until a mean weight of about 160 gm. was reached. Thereafter growth became progressively slower. The weight increases were accompanied by increasing plasma esterase levels as shown in figure 1. The line drawn was calculated by the method of least squares for a linear relation.

Following the first injection there was a considerable weight loss in all cases (table 1). Thereafter, the animals receiving 0.5 and 0.7 cc. per kg. on the whole maintained constant weight, regardless of whether the controls were gaining weight rapidly or slowly. The animals receiving 0.85 cc. per kg. lost weight during the entire course of the experiment. (Experiment 3B, table 1).

The plasma esterase levels of normal and experimental rats did not differ significantly up to the fourth day of the experiment. Using the *t*-test for significant differences (5) *t* was found to be between 0 and 0.8, *n* between 10 and 20, *P* > 0.4, in all experiments, except for 3B. In this most severely exposed group the plasma esterase levels on the fourth experimental day may be significantly below the control level (*t* = 1.3, *n* = 10, *P* < 0.3). As pointed out above, weight changes between the fourth and eighth days were quite small. Plasma esterase levels, on the other hand, showed a sharp drop during this interval; on the eighth and succeeding days the plasma esterase level was considerably lower than it had been on the fourth day. In fact, in all experiments the plasma esterase level at this time was found to be near 40% of the value observed in the normal controls (*t* ≈ approximately 5, *n* = 14 - 20, *P* < 0.01). This value was observed in all groups and for all dosages, within the limits of the biological variation of the several groups. Continuation of injections beyond the eighth day did not lead to further loss of cholinesterase activity of the plasma. In experiment 4, the rats treated with carbon tetrachloride were permitted to recover

for seven days after the last of five injections. At the end of this recovery period, body weight and plasma esterase activity had returned virtually to the values found for the control animals ($t = 0.9$, $n = 14$, $P < 0.4$).

It appeared desirable to obtain data concerning the degree of liver injury produced by the carbon tetrachloride in order to correlate impairment of liver

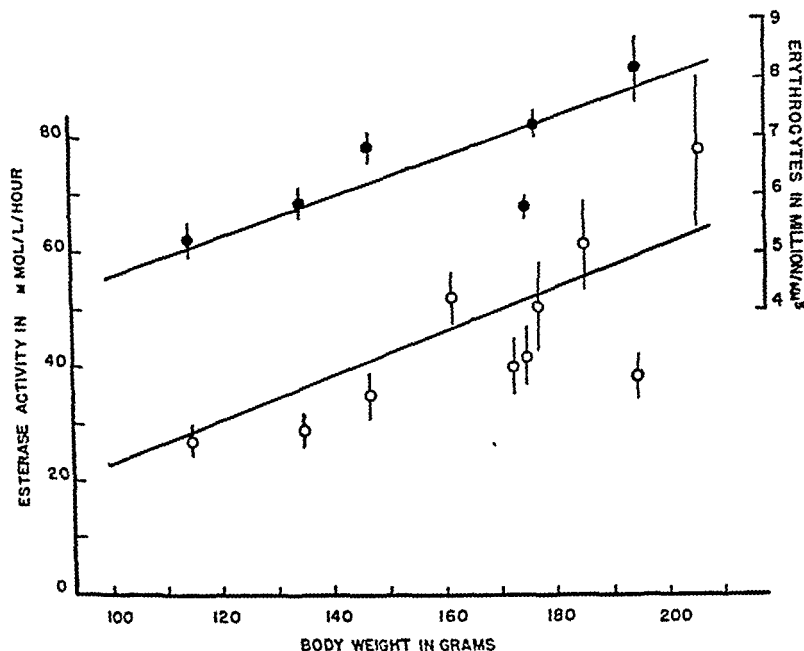


FIG. 1. THE CHANGES IN PLASMA CHOLINESTERASE ACTIVITY AND ERYTHROCYTE COUNT IN THE GROWING FEMALE ALBINO RAT
 ○—Cholinesterase activity.
 ●—Erythrocyte count.

The magnitude of the deviation of the mean for each point is indicated in the figure by a vertical line drawn through that point.

functions with the observed changes in plasma cholinesterase activity. Histological studies² revealed pronounced liver injury from the fourth day of the experiment on, increasing slightly in severity with prolonged exposure. Somewhat more extensive damage was seen in animals exposed to the higher doses than in those which received only 0.5 cc. per kg. with each injection. The lesions were mainly centrilobular, but some periportal infiltration was noted. The livers of the recovered rats of experiment 4 were reported normal.

An attempt was made to secure more quantitative data concerning changes in the functional state of the livers of the carbon tetrachloride treated animals.

² Dr. Benjamin Landing, Pathology Laboratory, Children's Hospital, Boston, Massachusetts, kindly performed the histological examination.

The decreased ability of such livers to inactivate evipal is illustrated by data obtained from experiment 4 of table 1. The relative evipal times obtained are represented in figure 2, together with the weight changes and a composite curve

TABLE 1

CONTROL RATS							CCl ₄ TREATED RATS						
Exp. no.	Day of exp.	No. of rats	Body wt. in g.		Mean	Stand-ard deviation of mean	No. of rats	Individual* and cumulative total dose of CCl ₄	Body wt. in g.		Mean	Stand-ard deviation of mean	% of control rats
			Mean	Range					Mean	Range			
1	1	20	168	140-200			20	0.7	172	146-222			
	4	10	173	140-210	40.3	±4.9	10	1.4	160	140-198	45.8	±5.2	115
	11	10	186	156-223	61.4	±7.8	10	3.5	163	130-204	25.3	±2.7	41
2	1	20	119	100-130			30	0.7	113	79-130			
	4	7	115	104-128	27.1	±2.5	10	1.4	108	80-130	26.2	±1.5	97
	8	6	135	116-147	29.2	±3.2	9	2.8	107	83-125	13.8	±2.9	47
	10	7	147	135-159	35.1	±3.9	10	3.5	115	83-141	14.6	±2.4	41
3A	1	15	179	159-201			15	0.5	181	170-198			
	4	5	179	159-201	41.7	±6.1	5	1.0	168	158-206	40.7	±10.0	97.5
	8	10	180	160-203	50.6	±8.1	10	2.0	156	140-167	19.2	±3.4	38
3B	1	15	179	159-201			18	0.85	176	148-194			
	4	5	179	159-201	41.7	±6.1	5	1.7	155	143-174	32.7	±4.1	79
	8	10	180	160-203	50.6	±8.1	13	3.4	140	124-155	22.7	±2.9	45
4	1	7	184	162-214			7	0.5	186	162-226			
	4	7	181	151-209			7	1.0	167	150-208			
	8	7	191	164-215			7	2.0	169	152-213			
	10	7	187	157-220			7	2.5	170	150-213			
	12	7	193	162-222			7		179	158-220			
	17	7	200	163-227	38.4	±4.3	7		196	168-232	33.3	±5.1	86
5	1	4	203	176-228			4	0.5	210	205-213			
	10	4	206	180-230	77.7	±14.7	4	2.5	201	194-223	41.5	±4.0	52
6	1	7	159	146-182			7	0.5	155	148-160			
	10	7	162	148-190	53.2	±4.9	7	2.5	136	120-150	24.9	±4.9	47

* The first figure appearing in this column in each experiment denotes the single dose injected every second day; the following figures are the accumulating total dose. The last figure for each experiment indicates the total dose given and is entered one day after the administration of the last injection.

showing the changes in plasma cholinesterase activity in animals injected with 0.5 cc. per kg. every other day.

The important features of the liver injury produced by the particular mode of

carbon tetrachloride administration chosen are indicated by both methods of assessing hepatic damage to be: a. The existence on the fourth day of the ex-

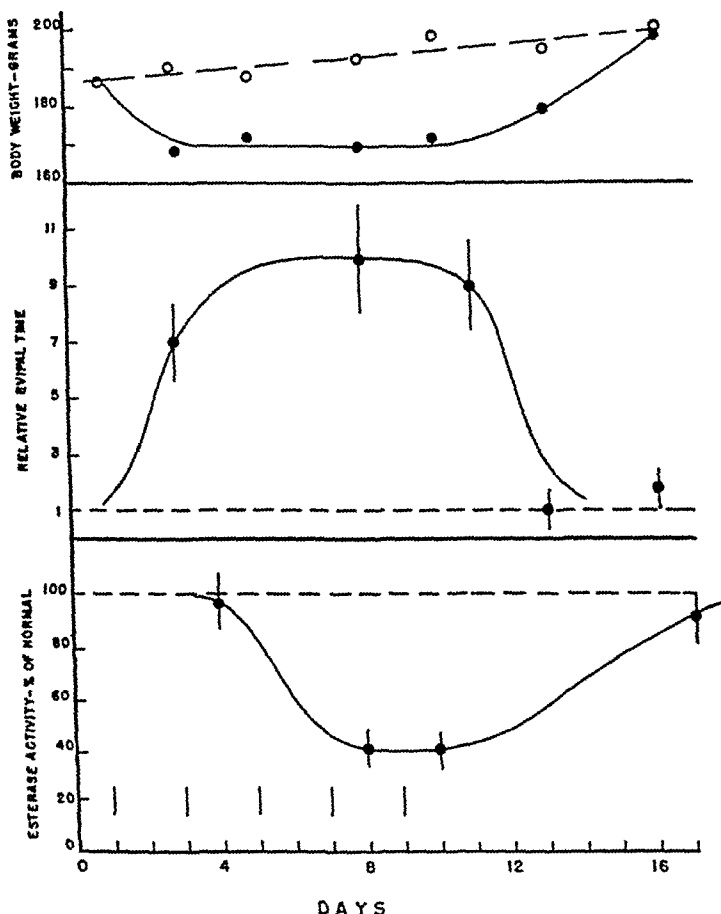


FIG. 2. THE EFFECT OF CARBON TETRACHLORIDE TREATMENT UPON THE PLASMA CHOLINESTERASE ACTIVITY, THE EVIPAL TIME, AND THE BODY WEIGHT OF FEMALE ALBINO RATS

Top—Body weight. O—normal, ●—carbon tetrachloride treated. Center—Evipal time (multiple of normal evipal time for that group). Bottom—Plasma cholinesterase activity in per cent of the normal plasma cholinesterase activity for that group. The magnitude of the standard deviations of the mean are indicated on the center and bottom graphs by the vertical marks immediately above each point. The vertical marks immediately above the x-axis indicate the days on which 0.5 cc. per kgm. of carbon tetrachloride was injected into the experimental animals.

--- normal control values, — values for experimental animals. The data for evipal time and body weight are taken from experiment 4, table 1. The plasma esterase plot is a composite curve representing data from experiments 3A, 4, and 6 of that table. Each of the values given represents between 5 and 10 experimental and as many control animals (see table 1 for precise figures for each point.).

periments of pronounced liver injury; b. between the fourth and the tenth days of treatment relatively small changes of the degree of liver injury; c. prompt

and complete recovery from a period of ten days of experimental carbon tetrachloride poisoning following withdrawal of the drug.

In many of the animals the kidneys were subjected to histological study. Only minor lesions were noted, especially of the proximal convoluted tubules. In the groups exposed to 0.5 cc. per kg. of carbon tetrachloride these lesions were hardly more frequent or more severe than those seen in normal rats of the same strain. With higher doses there is evidence of slight renal injury referable to the drug.

The changes in erythrocyte counts during growth in normal animals are represented in figure 1 together with the plasma esterase levels. No significant differences between the erythrocyte counts of normal and of injected rats of the same age groups could be observed.

In several experiments the fresh weights of livers, kidneys, and uteri of control and experimental animals were determined and compared. The groups employed were too small to permit valid generalizations from these data. In general, kidney and uterine weights reflected the growth—or failure to grow—of the various animals. By contrast, the liver weights of control and experimental animals in general were nearly equal, if anything the treated rats had somewhat heavier livers than the controls. Thus, the relative liver weights in all treated rats were considerably above the normal values.

DISCUSSION. It has been shown (6) that under the conditions chosen for the determination of plasma cholinesterase activity only about 10% of the activity measured can be due to the so-called "true" ("specific") cholinesterase, 90% being due to the ("pseudo") plasma cholinesterase. It is not yet possible to decide on the basis of extant information whether this 90% of the total activity is due to a single enzyme or to several, each contributing an important portion of the remaining activity. However, it has been shown that most of the simple esterases will split acetyl choline only slowly (7).

The observed decrease of plasma esterase activity might either be due to an actual decrease in the amount of this enzyme (or these enzymes) present in the blood plasma, or substances which are inhibitors of cholinesterase activity might appear in the plasma as a consequence of the carbon tetrachloride treatment (see e.g. (8)).

A direct effect of either carbon tetrachloride or of the products of its metabolism upon the determination can be excluded in view of the absence of changes in the esterase levels of animals on the fourth day of the experiment—i.e. after two injections of the solvent.

The presence of reversible inhibitors was excluded by determining the cholinesterase activity of the pooled plasma of rats (experiment 6) exposed for ten days to a level of 0.5 cc. per kg. of carbon tetrachloride using several dilutions of the plasma ranging from 1:5 to 1:20. The following results were obtained for cholinesterase activity of pooled plasma measured at the dilutions indicated:

1:5 —31.0 mMol/L/hour
1:10—29.4 mMol/L/hour
1:20—29.6 mMol/L/hour

The absence of any dilution effect (9) excludes the presence of a reversible inhibitor in amounts which would affect the conclusions to be drawn from these experiments.

The possibility was considered that irreversible inactivation rather than reversible inhibition might account for the low plasma cholinesterase activities of the carbon tetrachloride treated animals. The presence in plasma of excess amounts of substances capable of producing such an effect is unlikely because the incubation of normal rat plasma with pooled plasma of carbon tetrachloride treated rats (experiment 5) resulted in no deviation from strict additivity of the activities of the two plasmas—i.e. no activity was lost in the process of incubation:

Cholinesterase activity of normal rat plasma	85.3 mMol/L/hour
Carbon tetrachloride treated rats (pooled)	43.4 mMol/L/hour
A mixture of equal amounts of these	64.8 mMol/L/hour
Calculated for mixture if activities are assumed to be simply additive	64.4 mMol/L/hour

In order to directly test whether constituents of bile (either bile salts or bile pigments) might be capable of inhibiting plasma esterase a sample of dog's gall bladder bile was briefly heated to 80°C. to inactivate the small amount of esterase which is present in dog's bile. This sample was neutralized to pH 7.4, mixed with three volumes of normal rat plasma, and incubated over night at 38°C.

Cholinesterase activity of	
Plasma	40.3 mMol/L/hour
Bile (heated)	0.0 mMol/L/hour
Plasma after incubation with bile	41.2 mMol/L/hour

Thus, the plasma of rats treated with carbon tetrachloride does not appear to contain any important amounts of free substances capable of irreversibly inactivating plasma esterase; the presence of normal biliary constituents even in considerable amounts in the plasma of such rats cannot be responsible for the low cholinesterase activity of such plasma.

At present the most plausible interpretation of the observed changes in plasma cholinesterase activity appears to be that they represent changes in the actual amount of circulating enzyme.

Carbon tetrachloride was employed in these experiments as a liver poison. It is well known, however, that the liver is not the only organ which may be injured by this solvent. Especially the central nervous system and the kidney have been reported to be vulnerable to carbon tetrachloride. Central nervous system lesions have been reported only late in cases of protracted chronic poisoning by vapors. Under conditions such as those of the present experiments CNS damage need not be anticipated unless repeated anesthesia has been produced (10). The doses chosen, however, were such that no significant degree of CNS depression was observed at any time. As regards the possibility of renal lesions, the results of the histological examination already have been reported. Accordingly, either no effect at all, or only minimal lesions were produced even after prolonged exposure to carbon tetrachloride in this experiment.

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Thus, the effect of the carbon tetrachloride appears to be confined essentially to the liver, where anatomical and functional injury could be demonstrated.

The rise in plasma esterase levels during growth in the normal female rats is considerably faster than the reported rate of increase of total plasma proteins in comparable animals (12). This situation is not unique. It has been reported, (e.g. 13) that in human infants the rate of increase of total plasma proteins and of albumin is considerably slower than the rate of increase of the plasma globulins, and in particular the α and β globulins. Since present evidence (14) indicates that the plasma esterase in humans forms part of the globulin fraction distinct from γ globulin these data suggest that the changes in the human plasma cholinesterase activity may parallel those described in the growing rat.

Several workers (15) (16) (17) have commented upon a possible relation of plasma estrogen and progesterone levels with liver and plasma cholinesterase activities in the immature female rat. Since the liver has been shown to be involved in the elimination of these hormones (18) in the rat the possibility of an

TABLE 2

EXP.	DAYS	BODY WEIGHT		PLASMA CHOLINESTERASE ACTIVITY IN mMOL/L/HOUR					
		Control rats	CCl ₄ Treated rats	Control rats		Controls corrected	CCl ₄ treated rats		% of corrected controls
				Mean	St.D.M.		Mean	St.D.M.	
1	11	186	163	61.4	± 7.8	53.0	25.3	± 2.7	47
2	4	115	103	27.1	± 2.5	22	26	± 1.5	119
	10	147	115	35.1	± 3.9	25	14.6	± 2.4	59
3B	8	180	140	50.6	± 8.1	33	22.7	± 2.9	69

The values of this table are taken from table 1. Details concerning the experiments from which the figures are taken will be found there.

St.D.M. = Standard Deviation of Mean.

indirect effect of carbon tetrachloride administration was considered. However, such an effect should influence plasma esterase levels in a direction opposite to that actually observed.

Since the plasma esterase changes as the animals grow, and since there are weight differences between the experimental and the control rats, would it be more correct to compare animals of the same weight rather than animals of the same age? This is done in table 2 for three experiments, employing the esterase curve of figure 1 to correct the plasma esterase levels of the control rats to correspond to the weights of the experimental animals. It appears that even under these conditions the effect remains significant ($t = 3.0$, $n = 10-20$, $P < 0.01$). Furthermore, since the same results are obtained for rats which showed only slight weight losses compared to the control group, and in rats with very severe losses of body substance, the original comparison of animals of equal age appears justified.

The different time courses of weight and of plasma cholinesterase activities indicate that the observed plasma esterase changes do not reflect changes in the

amount of food taken in or retained. The greatest weight loss, and the least food intake in prolonged carbon tetrachloride poisoning are observed on the first and second days. Thereafter, the weight losses stop, and the food intake rises to approximate that of the control animals (cp. (20)). Administration of carbon tetrachloride in the doses here employed produces severe liver injury within 24 hours (19). On the fourth day of the experiments well established liver injury and dysfunction have been found. Yet, at that time no drop in plasma esterase has been observed. This appears only later, between the fourth and the eighth days. This lag may be only apparent due to the variation of animals which could prevent the detection of small differences in plasma esterase during the early days of an experiment. An initial contraction of the plasma volume might also forestall an early drop of the plasma esterase level. Such explanations fit poorly, however, with the sharp drop seen between the fourth and the eighth days. The following considerations may provide a more plausible explanation: It is well known that considerable cholinesterase concentrations are found in the liver. Several workers have observed a parallelism between the plasma and liver esterase concentrations (e.g. (21)). If the cholinesterase activity of the liver of normal rats is compared with that of animals treated for ten days with carbon tetrachloride (experiment 6), a much more pronounced loss of esterase is observed in the livers of the experimental animals than in their plasma.

Normal livers...	251.6 \pm 33.0 mMol/1000 gm. tissue per hour
Livers from carbon tetrachloride treated animals	53.0 \pm 11.6 mMol/1000 gm. tissue per hour

Compare the decrease to 21.1% of the controls for liver esterase with the decrease of the plasma cholinesterase activity to 47.2% in the same animals. These data are based on fresh weights of tissue, and—since there were no significant changes in total fresh liver weights—a pronounced decrease in total liver cholinesterase activity is indicated. Further, since these animals had been maintaining constant weight for about eight days at the time of killing, an acute depletion of liver protein due to nutritional causes appears quite improbable.

These observations suggest that the effect of carbon tetrachloride injury may be two-fold: it may impair the ability of the liver to produce the enzyme, and it may cause an outpouring of part of the stored esterase from the tissue into the blood stream. Such a dual effect would satisfactorily account for the lag between the appearance of liver damage and the drop in plasma esterase as well as for the very low liver esterase levels found following carbon tetrachloride treatment.

The apparent lower limit of the plasma cholinesterase activity in the present experiments is probably due to the narrow dose range which in combination with the wide range of variation of plasma cholinesterase activities precluded the observation of a dose-response relation. Furthermore, it appears possible that in the rat as in the dog (22) reduced plasma—and presumably liver—levels of various proteins stimulate the rate of production of new protein. Such an effect might well serve to further obscure a dose-response relation which might exist.

The present data do not yet exclude the possibility that extrahepatic sources of part or all of the remaining 40% of the plasma cholinesterase activity exist.

All of the above discussion has been predicated on the assumption that the phenomena observed must be accounted for in terms of a failure of the synthesis of the enzyme rather than in terms of an increased rate of destruction resulting from the carbon tetrachloride treatment. To date no evidence for such a mechanism has been obtained for any of the proteins which have been carefully investigated.

SUMMARY

Data have been presented concerning the changes in the acetylcholine splitting activity of rat plasma during growth and following the administration of carbon tetrachloride.

It has been concluded that the effects observed reflect changes in the amount of enzyme circulating in the plasma.

The drop of plasma cholinesterase activity following carbon tetrachloride injection is attributed to the liver injury produced by the poison, and is suggestive of a hepatic origin of the plasma enzyme.

The existence of a mobile reserve of (choline-) esterase in the liver has been suggested. The existence of such a reserve could explain the lag in the drop of the plasma cholin esterase activity as well as the very pronounced fall in the liver cholinesterase activity in carbon tetrachloride poisoning.

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THE PHARMACOLOGY OF PENICILLIC ACID

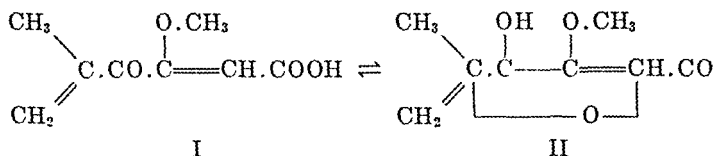
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Penicillic acid is the name given by Alsberg and Black (1) to a substance which they isolated while investigating the possible connection between the incidence of pellagra and mould deterioration of maize. They showed that it was a metabolic product of *Penicillium puberulum*, Bainier, a mould which may contaminate this cereal. Oxford and Raistrick (2) showed that the mould *Penicillium cyclopium*, Westling, also produced relatively large amounts of penicillic acid. *Penicillium suavolens*, *Penicillium thomii*, *Aspergillus ochraceus*, can all yield penicillic acid, the latter as much as 200 mgm. per litre of culture medium (3). It is soluble in cold water to about 2 per cent; it is readily soluble in hot water, alcohol, ether, benzene and chloroform and insoluble in cold light petroleum; it crystallises from light petroleum in the anhydrous form ($C_8H_{10}O_4$) and from water in the hydrated form ($C_8H_{12}O_5$); it is optically inactive and has one active hydrogen atom.

Birkinshaw, Oxford and Raistrick (4) from chemical evidence postulated the existence of penicillic acid in two tautomeric forms (γ -keto- β -methoxy- δ -methylene- Δ^a -hexenoic acid (I) and the corresponding $\Delta^{a\beta}$ - γ -hydroxy lactone (II)):



Oxford, Raistrick and Smith (5) and Oxford (6) showed that penicillic acid possessed antibacterial action against both Gram-positive and Gram-negative bacteria, inhibiting their growth in concentrations of 1 in 100,000 to 1 in 50,000. They also showed that penicillic acid, although it has a widespread bacteriostatic action, is particularly active against members of the Coli-Typhoid-Salmonella group of Gram-negative organisms, organisms on which penicillin has no appreciable effect.

Since penicillic acid is a naturally occurring substance, obtainable in pure crystalline form and of known chemical structure, an examination of its pharmacological properties has been undertaken (compare that for patulin; 7). A supply of impure penicillic acid was very kindly placed at my disposal by Sir Howard Florey, F.R.S.; it was recrystallised by Dr. H. R. Ing, who established its purity.

RESULTS. Toxicity. Alsberg and Black (1) state that penicillic acid kills mice on subcutaneous injection in a dose of 0.2–0.3 gm. per kgm. This corresponds to 5 mgm. per 20 gm. My observations indicated that the L.D.50 by this

route was 2.2 mgm. per 20 gm.; 60 mice were used. The intravenous L.D.50 was found to be 5.0 mgm. per 20 gm. (10 out of 20 mice), and the straight line relating log dose to mortality was steeper. When the slope of the line, b , was calculated by Gaddum's method (8), it was found to be 7.4 for subcutaneous injection and 13.3 for intravenous injection. Doses of 15 mgm. per 20 gm. or more when given intravenously caused convulsions terminating in death. No characteristic features such as the oedema caused by patulin were found *post mortem*.

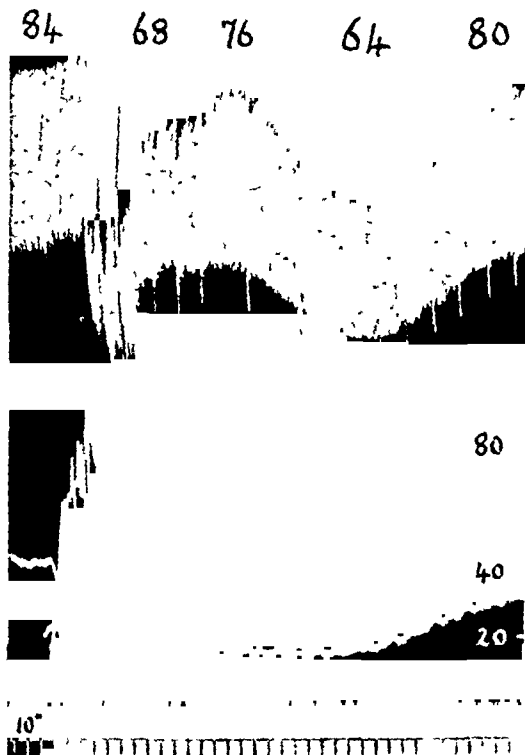


FIG. 1. RABBIT UNDER URETHANE

Upper record is respiration (Gaddum's method). At arrow penicillic acid injected, 30 mgm. per kgm. Note rise in blood pressure followed by fall. The respiration was slower and less deep. The numbers give the rate of respiration per min

When penicillic acid was given orally the mean lethal dose was about 12 mgm. per 20 gm. Although the number of mice used for each dose was less, the slope again appeared to be steep. When given 10 mgm. per 20 gm., all mice survived, and when given 14 mgm. per 20 gm. all mice died. The figures for toxicity were: (a) subcutaneous, 2.2 mgm. per 20 gm.; (b) intravenous, 5.0 mgm. per 20 gm.; (c) oral, 12.0 mgm. per 20 gm.

Penicillic acid is thus about 8 times less toxic than patulin by subcutaneous injection, 10 times less toxic by intravenous injection, and 17 times less toxic by mouth.

Action on the circulation. The blood pressure: In the rabbit under urethane the effect of an intravenous injection of penicillic acid at pH 7 was variable. After an initial dose of 10 mgm. per kgm. in a good preparation with a blood pressure of 75 mm., there was sometimes a transient fall followed after recovery by a further slight but prolonged fall. A repetition of this dose or the injection of twice this dose produced a sharp rise which passed off giving place to a fall. This effect is illustrated in figure 1. If the preparation was less vigorous, with a blood pressure between 40 and 50 mm., the rise was not seen; there was an initial fall, a recovery and a further prolonged fall.

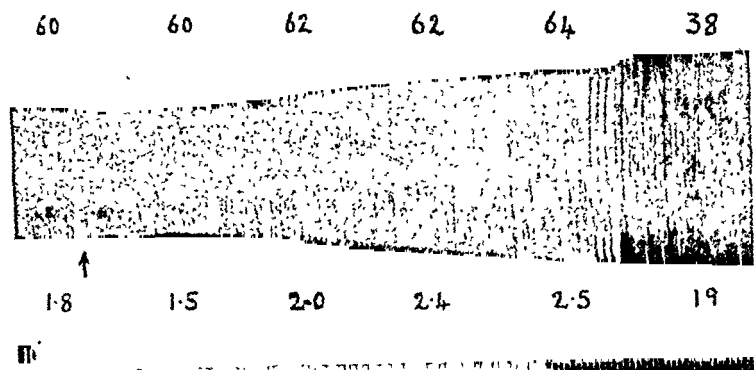


FIG. 2. PERFUSION OF FROG HEART THROUGH INFERIOR VENA CAVA

Record shows heart rate (above), amplitude, and output (figures below, cc. per min.). At arrow penicillic acid 10^{-3} perfused, containing frog blood (2 drops in 500 cc.). Note increase in output with increase of amplitude, and fall in output when the rate falls.

In the cat under chloralose the rise was less conspicuous, though more constant and was followed by a prolonged reduction in the blood pressure. In one experiment on a decerebrate cat both the rise and the fall were slight, though an injection made during the infusion of adrenaline produced a large fall.

In the spinal cat where the vascular tone was already low there was little effect; no appreciable rise or fall was seen.

The heart: When the frog's heart was perfused through a cannula tied into the inferior vena cava, the outflow being recorded from a cannula tied in one aorta, penicillic acid in concentrations of 10^{-3} , 4×10^{-4} and 10^{-4} caused a characteristic response. The first change was an increase in amplitude. The output per min. increased in some preparations and decreased in others. Increase was observed when the rate remained constant, while the decrease in output occurred when the rate dropped (see fig. 2). After some time the systolic height of contraction began to decrease, and simultaneously the diastolic relaxation became

incomplete. The heart began to beat irregularly and finally the ventricle stopped in systolic contracture while the atria and sinus continued beating.

The isolated rabbit's auricle when treated with penicillic acid in a concentration of 10^{-3} , also showed an increase in amplitude; after some time the amplitude

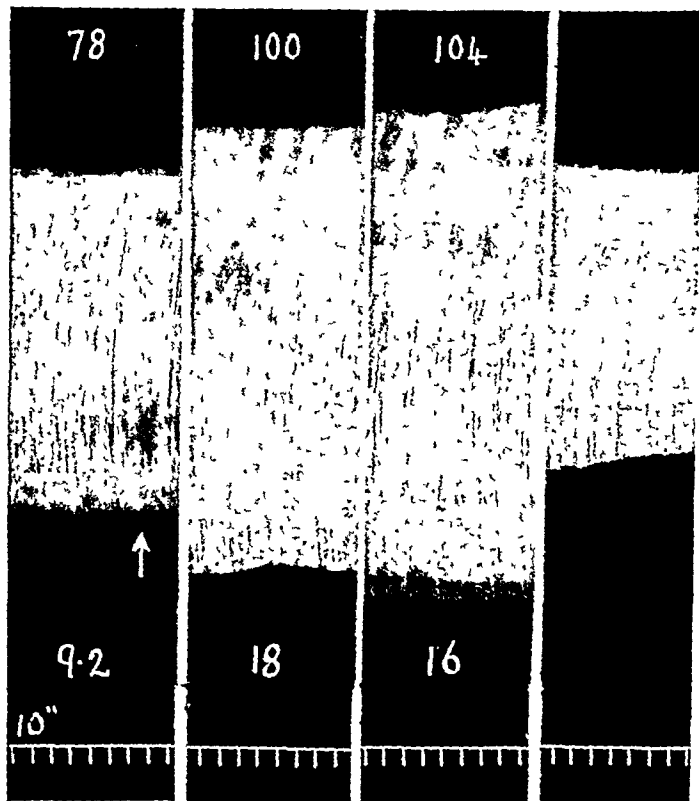


FIG 3 CAT HEART PERFUSED THROUGH AORTA AT 37°C

Figures above are rate per min. Figures below are outflow from coronary vessels. At arrow, perfusion begun with penicillic acid 3×10^{-4} . Note the augmentation of the amplitude and the great increase of the coronary flow.

decreased and finally the auricle passed into systolic contracture. In this preparation too, the rate gradually decreased.

The isolated cat's heart (Langendorff's preparation), on steady perfusion with concentrations of penicillic acid of 10^{-3} , 3×10^{-4} and 10^{-4} , first showed a slight transient depression in amplitude, followed by a large increase. After some time the amplitude decreased, the ventricles finally passing into systolic contracture. The rate first increased, but eventually decreased. There was a striking in-

crease in the coronary flow which persisted (see fig. 3). This greater flow caused a slight rise of temperature of the Ringer's solution reaching the heart which was probably responsible for the increase in rate.

The three preparations, the frog heart, the rabbit auricles and the cat heart perfused through the aorta, in each of which many experiments were made, thus showed that penicillic acid exerts a digitalis-like action on cardiac tissue. It causes an increase in amplitude without much change in rate, giving place after a time to cardiac irregularity and arrest in systole. The systolic contracture is irreversible. The effect was observed with high concentrations only and for this reason it seemed doubtful whether the digitalis-like property was sufficiently strong to be of importance. Experiments were therefore carried out to see whether, like strophanthin or one of the digitalis glycosides, penicillic acid would increase the work done by a partially damaged heart. The Starling heart-lung preparation of the dog was therefore used, for in it the output can be measured. Various substances have been tried to produce cardiac damage. Kuschinsky and Oberdisse (9) used Somnifen; in my experiments pentobarbitone was injected. The left auricular pressure was recorded and was observed to rise when 20 mgm., pentobarbitone was added to the venous reservoir and was also injected into the vena cava.

In three experiments the same result was repeatedly obtained when penicillic acid was added to the blood in the reservoir or injected into the vena cava in a dose of 100 mgm. There was a fall in the left auricular pressure (see fig. 4) but no increase in cardiac output. Thus the beneficial effect was too slight to be important. In one experiment 0.05 mgm. k-strophanthin was injected subsequently, and this caused the output to rise from 200 cc. to 756 cc. per min. in 20 min.

In one experiment a Morawitz cannula was tied in the coronary sinus, and the coronary flow was measured. The injection of 40 mgm. penicillic acid into the venous reservoir caused the flow from the sinus to increase from 60 cc. per min. to 96 cc. per min. in 17 min.

Lethal action on the frog and the guinea-pig: When penicillic acid was injected into the lymph sac of the frog, it caused systolic arrest of the heart in a dose of 7.5 mgm. per 20 gm. Digitalis leaf under similar conditions has this action in a dose of 10 mgm. per 20 gm. By slow intravenous injection into the guinea-pig, penicillic acid produced cardiac arrest in two animals in doses of 0.33 gm. and 0.83 gm. per kgm. Digitalis leaf under similar conditions has this action in a dose of 0.15 gm. per kgm. Hence the guinea-pig heart is much less sensitive to penicillic acid than the frog heart.

Pulmonary vessels: The injection of penicillic acid in the heart-lung preparation was followed by a disappearance of some of the circulating blood, some of which appeared to accumulate in the lungs. An experiment was therefore performed in which the lungs of a dog were perfused with defibrinated blood through the pulmonary artery. A cannula, which consisted of a tube broadening out above to 2 cm. diameter, was tied in the pulmonary artery so as to be vertical. A pump delivered blood into this tube through a rubber stopper and

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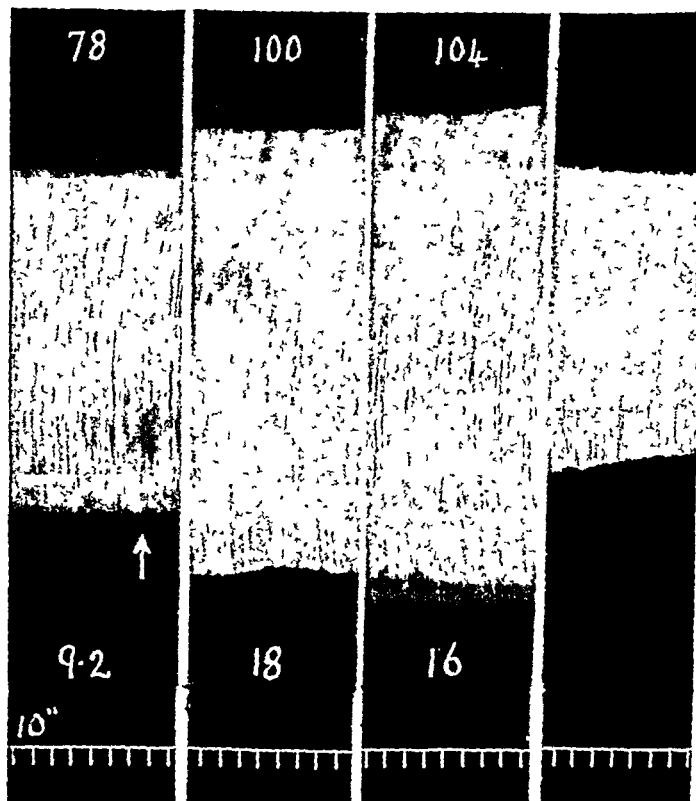


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the volume of air in the tube was recorded by a piston recorder (see fig. 5). When the pulmonary vessels constricted, as after the injection of adrenaline, the recording lever rose. Penicillic acid was seen to dilate the pulmonary arterial vessels.

Systemic vessels: (a) Rabbit's ear. The vessels of the rabbit's ear were perfused with Ringer's solution by the method of Gaddum and Kwiatkowski (10) through the carotid artery, and the outflow was recorded by Gaddum's drop timer. The smallest dose which had any effect was 2 mgm. which caused transient vasodilatation. Larger doses from 5-50 mgm. caused larger vasodilator effects (see fig. 6).

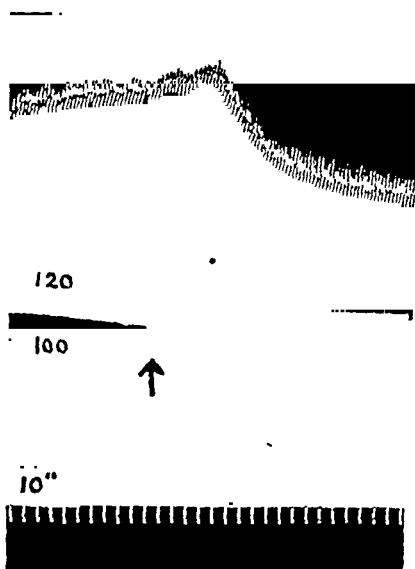


FIG. 4. DOG HEART-LUNG PREPARATION

Upper record left auricular pressure. Lower record aortic pressure. At arrow, 100 mgm. penicillic acid injected into vena cava, and 100 mgm. also added to venous reservoir. Note fall in left auricular pressure denoting increased ventricular contraction.

(b) Dog's hindleg perfusion. When the vessels of the dog's hindleg were perfused with defibrinated blood by a Dale-Schuster (11) pump, doses of penicillic acid from 2-50 mgm. caused vasodilatation, shown by a fall in perfusion pressure and increased venous outflow. This vasodilatation occurred in two distinct stages, 1) an initial effect of short duration immediately after the injection, and 2) a gradual prolonged effect due to the persistence of penicillic acid, and proportionate to its final concentration in the system. Both in the rabbit's ear preparation and in the dog's hindleg, the vasodilator effects were readily modified by adrenaline. The presence of a weak adrenaline tone produced for example by 2×10^{-8} in the Ringer's solution perfusing the rabbit's ear increased

the dilator action of penicillic acid. When, however, there was a more powerful adrenaline tone, 5×10^{-8} , the dilator action was reduced.

(c) Effect of capillary permeability. The vessels of the frog's hind limbs were perfused with Ringer's solution containing 0.25 per cent gelatine using a method described by Hijman and Chambers (12), by which the gradual increase in weight due to the slow onset of oedema is recorded. The rate of oedema formation is indicated by the slope of the tracing.

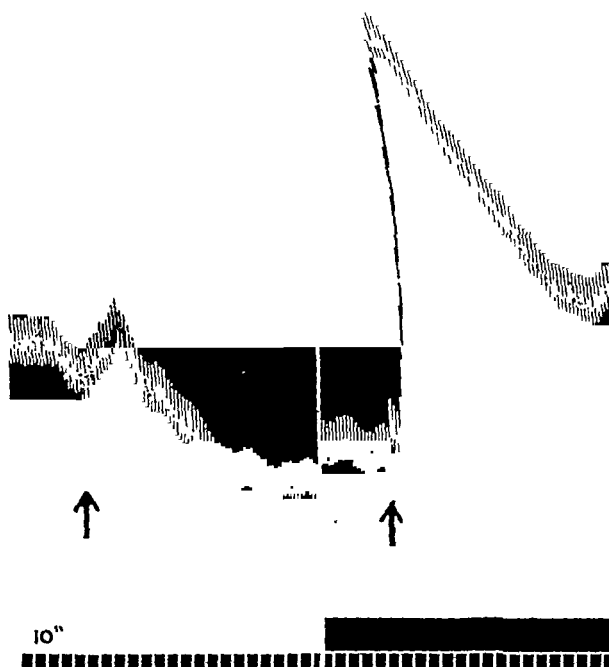


FIG. 5. PERFUSION OF DOG LUNGS

At first arrow, 50 mgm. penicillic acid injected into blood going to pulmonary artery. Note fall of pressure indicating pulmonary dilatation. At second arrow 5 μ g. adrenaline was injected. Note pulmonary vasoconstriction.

On perfusion with 1 in 1000 penicillic acid, the slope of the tracing gradually fell, indicating a decreased rate of oedema formation. With a concentration of 1 in 100 penicillic acid this decreased rate of oedema formation was very great, and corresponded approximately in effect to that produced by adrenaline in concentration of 10^{-8} (see fig. 7).

Action on smooth muscle. Intestine: When a loop of rabbit intestine was suspended in a bath of 50 cc., the addition of penicillic acid in amounts up to 40

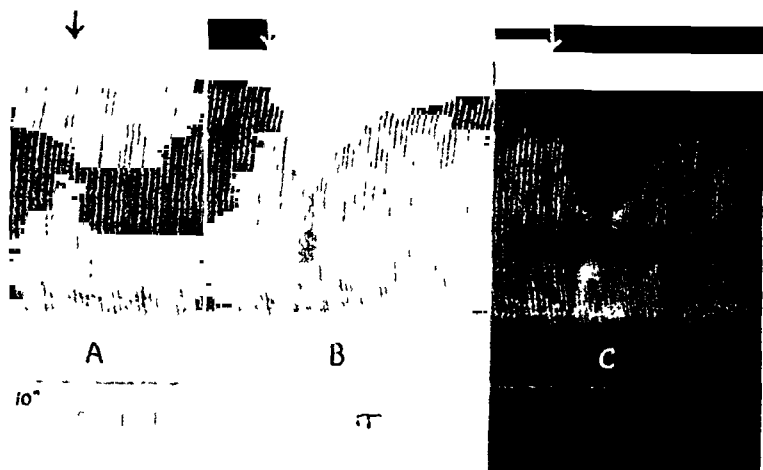


FIG. 6. PERFUSION OF RABBIT EAR VESSELS

Outflow record by Gaddum's drop timer. Injection of penicillic acid. At A, 2 mgm., at B, 10 mgm., and at C, 50 mgm. The effects indicate vasodilatation.

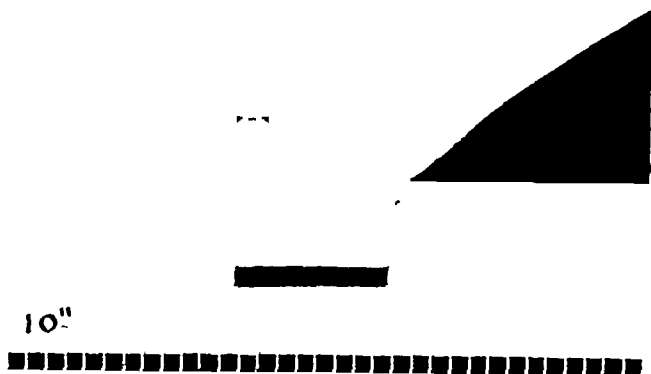


FIG. 7. HJMAN-CHAMBERS PREPARATION TO SHOW RATE OF OEDEMA FORMATION

Note reduction of oedema produced by adding penicillic acid 10^{-2} to the Ringer for the period of the white block only.

mgm. had no effect on the pendular movements or tone. When the contractions of the intestine *in situ* were recorded by means of a balloon inserted into the duodenum of a cat anaesthetised with chloralose, different effects were observed

in different experiments. In the experiment illustrated in figure 8, the injection of 100 mgm. penicillic acid caused a sharp rise of blood pressure accompanied by an inhibition of the intestinal movements for several minutes. In a similar experiment there was no rise of blood pressure and no inhibition, indeed after 4 min. a great increase of intestinal movement was seen. These results suggest that the rise of blood pressure shown in figure 8 was due to stimulation of the vasomotor centre, and the resulting outflow of sympathetic impulses caused intestinal inhibition also. In the second experiment there was no rise of blood pressure and the inhibition was absent. A lack of sufficient penicillic acid made

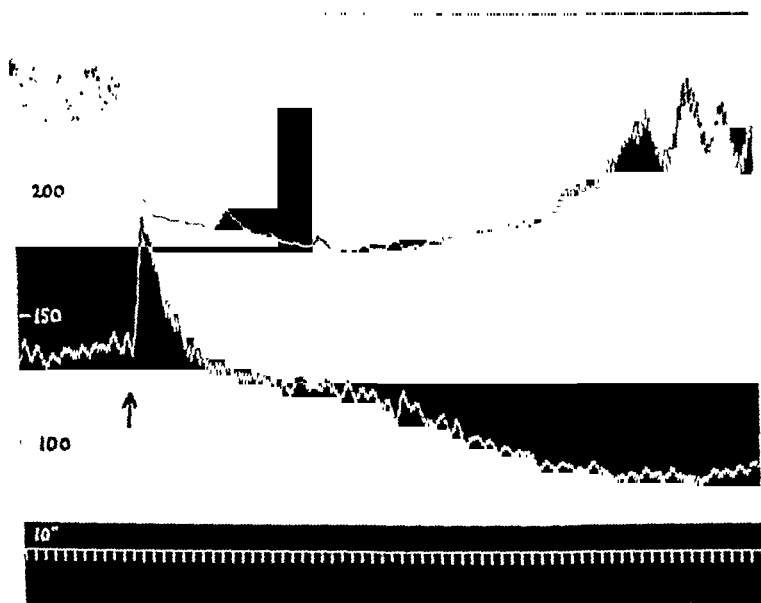


FIG. 8. CAT, CHLORALOSE

Intestinal movements recorded by balloon in duodenum. At arrow, 100 mgm. penicillic acid injected into jugular vein. Note rise of blood pressure accompanied by inhibition of intestinal movements. Note later fall of blood pressure.

it impossible to investigate this further. Penicillic acid was observed to have no action on spleen volume either in the spinal cat or in the cat under chloralose.

Respiration. Experiments were carried out in rabbits and in cats in which the respiration was recorded by Gaddum's method. In a rabbit under urethane in which an injection of 30 mgm. per kgm. penicillic acid caused a rise of blood pressure followed by a fall, the respiratory rate decreased from 84 to 68 (see fig. 1). This depression may have been secondary to the rise of blood pressure, for in those experiments in the decerebrate cat in which the injection of 100 mgm. penicillic acid caused no rise of blood pressure, the respiratory rate increased from 16 to 32 per min. There was not much effect on the depth of respiration.

Action on water diuresis. Penicillic acid was found to have an antidiuretic effect in rats. Two groups of four rats each were taken for each experiment. All were given 5 cc. of warm tap water per 100 gm. by stomach tube. Penicillic acid was injected subcutaneously into one group. The observation period was 4 hours, during which the amount of urine excreted was measured. On the following day the experiment was repeated, but the previous control group was injected while the previous injected group acted as control. This procedure was repeated using different doses of penicillic acid ranging from 0.1–8.0 mgm. per 100 gm. rat. The result of one experiment is shown in figure 9 in which the rats were injected

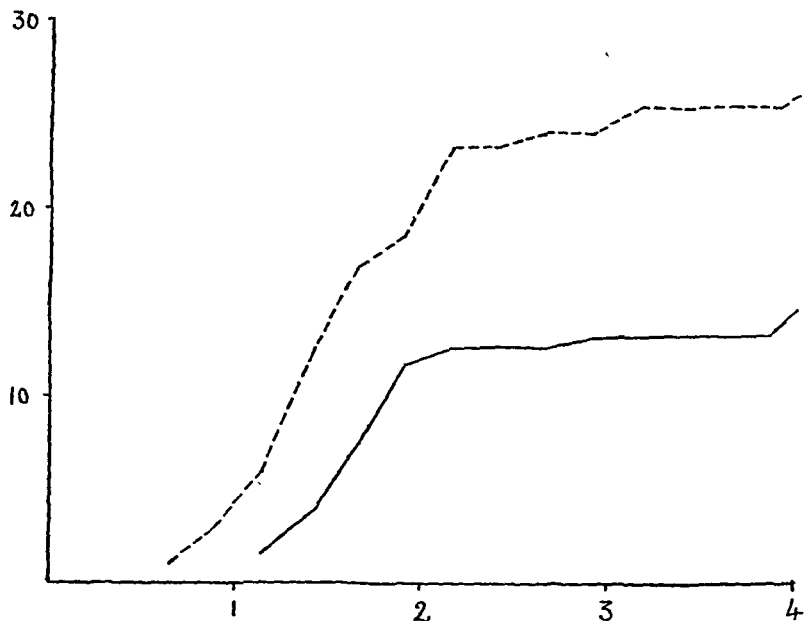


FIG. 9. Abscissae—time in hours. Ordinates—urine volume in cc. Dotted line shows output of urine by 4 rats when given 5 cc. water per 100 gm. by mouth. Continuous line shows output when 2 mgm. per 100 gm. penicillic acid was injected subcutaneously. The observations were made in the form of a "cross-over" test.

with penicillic acid in a dose of 2 mgm. per 100 gm. The volume of water excreted in 4 hours when the rats received this injection was 54 per cent of the volume they excreted when they received no penicillic acid. Even a dose of penicillic acid so low as 0.1 mgm. per 100 gm. produced some antidiuretic effect. 87 per cent of the water being excreted. Figure 10 shows the relation between the dose and the antidiuretic action. Since the lethal dose by subcutaneous injection was 11.0 mgm. per 100 gm. (in the mouse) it is evident that an antidiuretic action was exerted by as little as 1 per cent of the lethal dose.

The antidiuretic action might have been due to a delay in the absorption of water from the alimentary canal; to test this, experiments were carried out on

rabbits anaesthetized with urethane in which a cannula was tied into the bladder. A diuresis was produced by an intravenous infusion of 40 cc. saline, and the penicillic acid was injected intravenously. A dose of 10 mgm. per kgm. regularly produced a drop in the urine output followed by a rise. The initial drop in one experiment was from 28 to 16 drops per min. about 3 min. after the injection; then the rate rose to 66 drops per min. during the next 4 min. The blood pressure fell by about 20 mm. when the injection was made, but this fall had passed off in 0.5 min. When the dose of 10 mgm. per kgm. was repeated there was a rise of blood pressure lasting 3 min. At the end of 2 min. the urine output fell from 46 to 4 drops per min. The antidiuretic effect occurred both when the blood

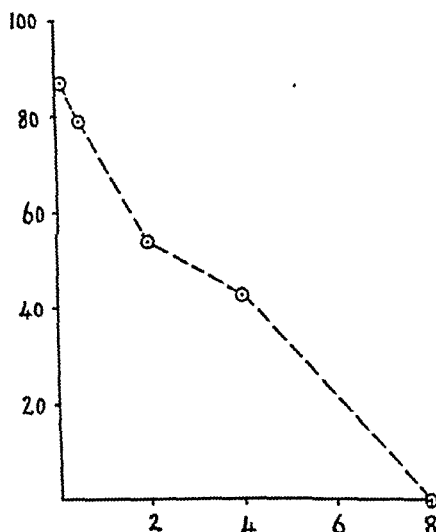


FIG. 10. Ordinates—percentage of water excreted in 4 hours, taking the volume of water excreted when no penicillic acid was injected as 100. Abscissae—dose of penicillic acid in mgm. per 100 gm. which was injected subcutaneously into each rat at the time the water was given. See text.

pressure fell and when it rose, and was therefore unlikely to be due to the blood pressure change.

Kidney perfusion. Some experiments were also carried out in which the kidney of a dog was perfused with defibrinated blood, and the blood reoxygenated by passing it through the lungs. The perfusion was begun at the moment the natural circulation stopped. The pressure in the renal artery was prevented from rising excessively by putting a side piece in the tube carrying blood to the renal artery, the side piece leading to an artificial resistance of the pattern used in the Starling heart-lung apparatus. The resistance was set at a level of about 130 mm. and the pressure in the renal artery could not rise above this. The effect of injecting into the blood flowing into the renal artery 20 mgm. penicillic acid was to reduce the urine flow from 28 to 20 drops per min. The injection of 50 mgm.

increased the flow at first from 25 to 38 drops per min., and then decreased it to 16 drops per min., after which it increased once more to 38 drops per min. The injection of penicillic acid dilated the renal vessels causing an increased blood flow, and this change appeared to be responsible for the increased urine flow. Thus there was a double effect, the antidiuretic effect being superimposed upon a diuretic action due to the increased blood flow. Later in this experiment the injection of 100 mgm. penicillic acid produced an uncomplicated increase of flow. The result, however, made it probable that the antidiuretic action was exerted locally on the kidney and not elsewhere.

Action on cilia. Since patulin was found to arrest the motion of a cat's trachea in a 1 in 10,000 solution, penicillic acid was also tested. Even in 1 in 100 solution, there was no indication that penicillic acid had any effect.

Discussion. The chief effects of penicillic acid on the circulatory system, i.e., the digitalis-like action on the isolated heart and the vasodilator effect on blood vessels, can be explained if it is assumed that penicillic acid acts pharmacologically as an unsaturated lactone.

Penicillic acid shares this cardiotoxic property with a large number of chemical substances which contain an unsaturated lactone ring in the molecule, e.g., cardiac glycosides, toad poisons etc. Penicillic acid may exist as a $\Delta^{\alpha\beta}$ -unsaturated lactone, and it is interesting to record that Paist, Blout, Uhle and Elderfield (13) present chemical evidence to suggest that the cardiac aglycones of the digitalis-strophanthus group may be more satisfactorily formulated as $\Delta^{\alpha\beta}$ -unsaturated lactones than as $\Delta^{\beta\gamma}$ -lactones as hitherto accepted. It should be noted, however, that Kraye et al. (14) showed that unsaturated lactones with the $\Delta^{\beta\gamma}$ -configuration are more active on the perfused isolated frog's heart than those with the $\Delta^{\alpha\beta}$ -configuration.

Ascorbic acid, which is an unsaturated lactone, also exhibits this cardiotoxic effect (15), but Kraye, Linstead and Todd (16) have shown that in this case the action is due to the presence of hydrogen peroxide in the perfusing fluid, formed during the dehydrogenation of the ascorbic acid, and that if this hydrogen peroxide is destroyed as rapidly as it is formed, the cardiotoxic effect is not produced. As suggested by these authors, 2 drops of frog's blood was added to 500 cc. of the perfusing fluid containing 1 in 1000 penicillic acid, in order to see whether penicillic acid, *per se*, exerted its cardiotoxic effect. The cardiotoxic effect was still obtained. This effect is produced on the heart in frogs, rabbits and cats, but only feebly in the dog's heart-lung preparation.

On the blood vessels of the dog's hind limb and rabbit's ear the only effect of penicillic acid is vasodilatation. This vasodilator response is altered by adrenaline. If adrenaline is present in a low concentration, the effect is magnified, while if it is present in a high concentration, it prevents the vasodilator effect. Penicillic acid causes vasodilatation of the coronary and pulmonary vessels.

This vasodilator effect of penicillic acid in the anaesthetised animal is shown by a fall in pressure, which is maintained, evidently due to the persistence of penicillic acid in the system. Chen *et al.* (17) found that unsaturated lactones cause a fall in blood pressure in anaesthetised cats. Accordingly, penicillic acid ap-

pears to act pharmacologically, on the heart and blood vessels, as an unsaturated lactone. This conclusion is, however, not in agreement with the chemical evidence of Birkinshaw, Oxford and Raistrick (4) which suggests that penicillic acid, at the pH of the body fluids must exist largely in the open-chain form. In anaesthetised and decerebrate animals, penicillic acid often causes a rise in blood pressure preceding the prolonged fall due to the direct vasodilator effect. This pressor effect appears to be due to stimulation of the vasomotor centre, leading to a general discharge of sympathetic impulses. The evidence in favour of such a suggestion is that in the cat under chloralose when penicillic acid causes a rise of blood pressure, there is a simultaneous inhibition of intestinal tone, but when there is no rise of blood pressure, there is no such inhibition. In the spinal cat penicillic acid does not raise the blood pressure.

In normal rats, and in anaesthetised rabbits and cats, penicillic acid causes an antidiuretic effect. This effect is not secondary to the effect on blood pressure, and is observed in the isolated perfused kidney of the dog. In the Hijman-Chambers frog preparation, penicillic acid exerts an action in inhibiting oedema formation. Since urine secretion across the glomerular membrane may be regarded as a transudation to the exterior, it seems possible that the property which penicillic acid possesses of reducing urine flow and the property of diminishing oedema formation, may be one and the same; penicillic acid reduces oedema formation by reducing capillary permeability in the frog's hind limbs, and reduces urine formation by reducing the permeability of the glomerular capillaries. If this is true the antidiuretic effect of penicillic acid must have a different mechanism from the antidiuretic effect of patulin, which is a substance shown to increase oedema formation.

SUMMARY

1. Penicillic acid is derived from *Penicillium puberulum* and other moulds. Its structure is known. A sample of crystalline penicillic acid has been examined pharmacologically.
2. It has a digitalis-like action on the heart of the frog, the rabbit auricle, the perfused cat's heart, and a very weak action in the heart-lung preparation of the dog. This activity suggests that it exerts its effect in the lactone form.
3. It has a dilator action on systemic blood vessels including the coronary vessels, and on the pulmonary vessels. When injected into the whole animal it often causes an initial rise of blood pressure probably due to stimulation of the vasomotor centre; the rise is absent in the spinal animal. This initial rise is followed by a prolonged fall.
4. It has an antidiuretic action which is detectable in rats after the subcutaneous injection of as little as one per cent of the L.D.50. This action can be seen on the isolated perfused kidney of the dog. This antidiuretic action may be due to a diminished permeability of the glomerular capillaries, since penicillic acid causes a diminished permeability of capillaries elsewhere.

(This work was done while the author was on leave of absence from the Department of Pharmacology, University College, Dublin.)

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HEMOLYTIC ANEMIA AS A MANIFESTATION OF PARAPHENYLENE-DIAMINE TOXICITY*

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The use of dyes containing paraphenylene-diamine on human hair, eyebrows and lashes, has caused severe dermatitis, dermatophthalmitis and systemic poisoning (1, 2). The subcutaneous injection of this chemical into rabbits and cats causes a characteristic edema of the head, which Tainter and Hanzlik (3) have shown to be due probably to increased capillary permeability. Macrocytic anemia in humans has been reported following the use of hair dyes containing paraphenylene-diamine (4); and two deaths, accompanied by liver damage, jaundice, and anemia seem to have been caused by hair dyes containing phenylene-diamine.

The present investigation was undertaken to study the toxic actions that might be produced by the sub-cutaneous injection of a supposedly sub-lethal dose of paraphenylene-diamine into dogs, and to see whether any anemia would be produced thereby.

PROCEDURES. Six normal dogs were injected subcutaneously with paraphenylene-diamine dihydrochloride in a dose of 30 mgm. per kgm. of body weight. Only a single injection was given to each dog, but observations were made on the animals before, and at frequent intervals after the injection. These observations included the search for manifest edema, erythrocyte counts, hemoglobin determinations (Hellige), readings of the icteric index, and the counting of reticulocytes on dried films of blood stained with cresyl blue.

RESULTS. Within a few hours following the injection of paraphenylene-diamine dihydrochloride, all six of the dogs showed some form of localized edema. The edema occurred in the face of one dog, in the throat and neck of a second, and in three of the feet of a third dog. In the other three dogs, the eyelids became very edematous, being swollen shut in two of the animals.

All dogs ultimately showed marked hyperemia and signs of irritation of the tissues around the eyes, which persisted for several days, in some cases. A large corneal ulcer developed on the eye of one dog, which was first noticed one month from the time of injection, and now seems to be permanent.

Two of the dogs died, one on the fifth day and the other on the tenth day following the injections of paraphenylene-diamine. Each dog showed signs of mechanical obstruction to breathing, and a loss of considerable fluid from the mouth, which led us to make a diagnosis of pulmonary edema. Neither of the dogs which died, showed any significant degree of anemia while they lived; and they both had marked hemoconcentration just before death.

The four dogs that survived indefinitely, all developed significant anemias early, i.e., within 48 hours following medication with paraphenylene-diamine.

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Figure 1 shows the changes in the erythrocyte counts of these dogs. They were reduced, sooner or later, by from 23 to 54 per cent of their original values, and remained subnormal for variable periods of time, ranging from 5 to 30 or more days. Hemoglobin values varied in a manner, generally proportionately to the red cell counts.

Icteric indices, observed on the blood of the two dogs having the most marked anemia, increased up to values of 40 and 42 (on the fourth day after medication). An increase in reticulocyte percentage, accompanied by an increase of nucleated red blood cells, was observed in one dog on the fourth day. This dog (represented by solid dots in figure 1) showed a maximal reticulocytosis of 8.6% (and

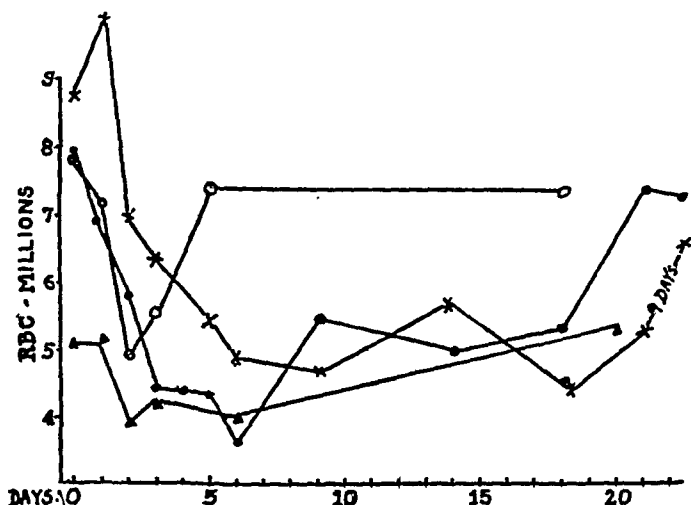


FIG. 1. ERYTHROCYTE COUNTS ON FOUR DOGS SHOWING THE PRODUCTION OF ANEMIA BY PARAPHENYLENE-DIAMINE

Each dog received a single subcutaneous injection of paraphenylene-diamine dihydrochloride (30 mgm./kgm.) at zero time.

0.4% blasts) on the tenth day, and his reticulocytes gradually diminished to a normal value of 0.4% on the twenty second day, by which time the anemia had disappeared. Observations on another dog indicated that a maximal reticulocytosis of 4.2% occurred on the eighteenth day.

DISCUSSION. The rapidity of the development of anemia following the injection of paraphenylene-diamine, as well as the early rise of icteric index, lead us to believe that the anemia is due to an accelerated rate of destruction of erythrocytes. The observation of significant reticulocytosis within four to ten days following administration of the drug lends support to a hemolytic mechanism, and also indicates that depression of bone marrow activity probably is not involved.

Whether the hemolysis of cells is caused by paraphenylene-diamine itself, or to some oxidation product of this substance, or to some other substance—we cannot say. Paraphenylene-diamine has a chemical structure that is somewhat like those of aniline and phenyl-hydrazine, and it is known that it easily oxidizes to quinone-diimine (7). There is also the possibility that the liver may be damaged by paraphenylene-diamine, and that some hemolytic substance consequently is formed or escapes detoxification.

CONCLUSIONS

The administration of a single subcutaneous injection of 30 mgm./kgm. of paraphenylene-diamine dihydrochloride to each of six dogs caused:

1. Localized edema in variable parts of the body.
2. Signs of irritation of the eyes and eyelids in all animals.
3. The deaths of two dogs, at five and ten days following the injection.
4. The development of acute hemolytic anemia in the four dogs which survived.

The anemias persisted for five to thirty days, and probably were *not*, in any case, due to depression of erythropoiesis.

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THE ANTAGONISM OF TETRAETHYLAMMONIUM AGAINST HEART DEPRESSANTS¹

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It has been known for a long time that some quaternary bases, e.g. tetramethylammonium (M 4 N), have a muscarine-like action on the frog's heart whereas others, e.g. tetraethylammonium (E 4 N), are not only devoid of this action but even antagonize it (1). This antagonism was interpreted many years ago by Schueller (2) and Kuelz (3) as due to an atropine-like action of E 4 N, and more recently, by Clark and Raventos (4) as due to competition for a common receptor because of the similarity of the molecular structure of the agents involved. It seemed of interest to investigate whether or not E 4 N antagonizes on the heart the effect only of substances of closely similar molecular structure.

The experiments were performed on isolated frogs' hearts (*Rana pipiens*) attached to a Straub cannula. The Ringer solution used had the following composition: 0.65 gram of NaCl, 0.01 gram of KCl, 0.01 gram of CaCl_2 , 0.02 gram of NaHCO_3 , and water to make 100 cc. solution. The drugs used were: tetramethylammonium chloride and tetraethylammonium bromide (Eastman Kodak), pilocarpine hydrochloride (Merck), acetylcholine hydrochloride (Hoffmann La Roche), arecoline hydrobromide (Fisher).

First, it was determined whether or not E 4 N has an action by itself on the frog's heart since reports concerning this question could not be found. For this reason hearts were used which had become a little hypodynamic by repeated washings with Ringer solution. This procedure was necessary since the fresh heart as a rule beats optimally. The action, therefore, of augmentor agents in this state is often absent or inconspicuous. If applied to a slightly or strongly hypodynamic heart, E 4 N 10^{-4} does not produce any visible changes. E 4 N 10^{-3} sometimes produces a slight increase of heart contractions and rate within about 2-3 minutes which in general wears off after a short while. Higher concentrations, as E 4 N 2×10^{-3} , cause an immediate strong increase similar to that produced by epinephrine. Recently, Acheson and Moe (5) found an augmentory action also on the mammalian heart.

In regard to the antagonistic action of E 4 N the results of former investigators, according to which the muscarine-like action of quaternary bases, such as M 4 N and acetylcholine, is antagonised (1, 4), could be confirmed. The antagonism takes place if E 4 N is applied previously or if applied when the muscarine-like action has reached its maximum. A strongly effective dose of acetylcholine 10^{-8} is almost entirely antagonised by E 4 N 10^{-4} (fig. 1); a much less effective dose of M 4 N 10^{-5} , by E 4 N 10^{-3} . These doses of E 4 N are easily washed out; in general it is sufficient to wash the heart just once or twice. After washing,

¹ Aided by grants from the Rockefeller Foundation and the Dazian Foundation.

acetylcholine (fig. 1) and M 4 N again produce their full effect. The fact that E 4 N is easily washed out indicates that it is loosely attached to the heart. The same holds true for acetylcholine in contrast to M 4 N.

Furthermore, the efficiency of E 4 N in antagonising the action of other vagomimetic drugs as pilocarpine and arecoline was investigated. The structure of these bases is very different from that of the quarternary bases mentioned above. They are tertiary bases.

1. 2. 4. 3. 4. 1. 2.

FIG. 1. 1. RINGER; 2. AcCh 10^{-6} ; 3. E 4 N 10^{-4} ; 4. E 4 N 10^{-4} + AcCh 10^{-6}

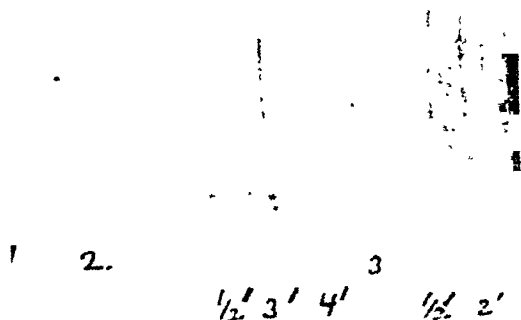


FIG. 2. 1. RINGER; 2. PILOCARPINE 10^{-5} ; 3. PILOCARPINE 10^{-5} + E 4 N 2×10^{-3}

The effect of a small effective dose of pilocarpine is slightly but definitely antagonized by E 4 N 10^{-3} , and completely by E 4 N 2×10^{-3} (fig. 2), a concentration which as previously mentioned strongly increases the heart beats. This antagonism is not specific for E 4 N since increased heart contraction, e.g. caused by adrenaline (fig. 3), regularly antagonizes the action of heart depressants. E 4 N proved to be a much more effective antagonist against arecoline. Here an intensive effect was not removed by repeated washings of the heart with Ringer solution but was entirely antagonized by E 4 N 10^{-3} , which by itself does not increase the heart beats (fig. 4).

Further experiments dealt with the question of whether E 4 N, in concentra-

tions devoid of an action by themselves, could also antagonize the action of other depressants of the heart action. It was found that such concentrations were inactive in heart depression caused by anoxia, by calcium deficiency, and by narcotics (pentobarbital sodium, chloralhydrate) but highly efficient against the depression caused by increasing the potassium concentration of the Ringer solution to 0.04 to 0.06% KCl. The depression caused by such a solution increases with repeated applications (fig. 5). In other words the heart becomes more sensi-

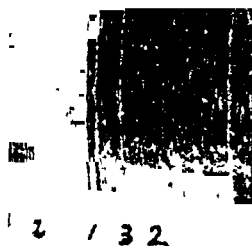


FIG. 3. 1. RINGER; 2. AcCh 2×10^{-8} ; 3. ADRENALINE 10^{-8}

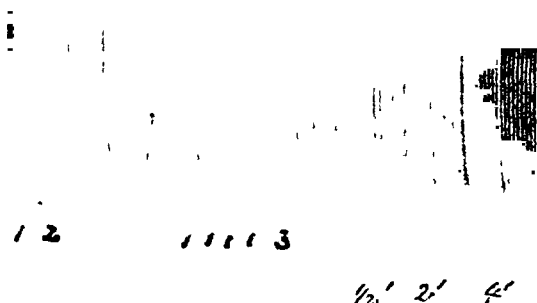


FIG. 4. 1. RINGER; 2. ARECOLINE 10^{-5} ; 3. ARECOLINE $10^{-5} + E 4 N 10^{-3}$

tive in time exactly as it does to repeated doses of acetylcholine. The cause of this sensitization may be a gradual loss of protective substances from the cell surfaces as a result of repeated washing since Clark (7) proved that continued washing of the heart with Ringer solution eventually led to a hypodynamic state which was overcome by re-adding substances lost by the washing. These proved to be of lipid character.

Figures 5, 6, and 7 demonstrate the antagonism of E 4 N against the effect of



FIG. 5. 1. RINGER; 2. RINGER KCl 0.06%; 3. E 4 N 10^{-3} ; 4. RINGER KCl 0.06% + E 4 N 10^{-3}

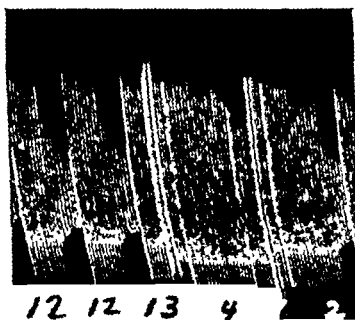


FIG. 6. 1. RINGER; 2. RINGER 0.06% KCl; 3. E 4 N 10^{-3} ; 4. RINGER 0.06% KCl + E 4 N 10^{-3}

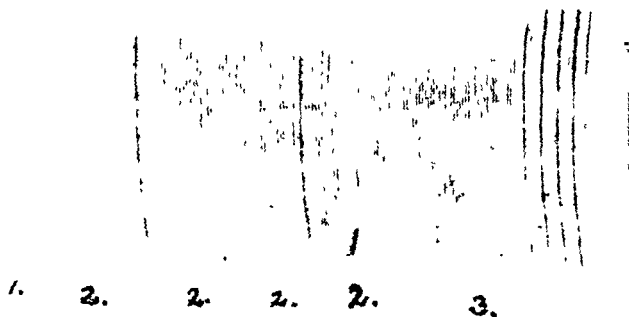


FIG. 7. 1. RINGER; 2. RINGER 0.04% KCl; 3. RINGER 0.04% KCl + E 4 N 10^{-3}

potassium rich solution. While in fig. 5, E 4 N by itself had no increasing action on the heart, in fig. 6 this same substance had a slight initial effect. The potassium, however, was not applied before the increasing effect of E 4 N had worn off. Figure 7 shows that the effect was antagonized when E 4 N was applied after the action of the potassium rich solution had developed.

DISCUSSION. In regard to the antagonism of E 4 N against the quarternary bases, M 4 N and AcCh, Clark's view (4, 13) is acceptable that it is due to competition because of the close similarity of their molecular structure. As to the antagonism of E 4 N against pilocarpine and arecoline, which are of different molecular structure, it has to be said that close similarity of molecular structure apparently is not a necessary prerequisite for competitive inhibition. Quite recently, for instance, it was proven experimentally that not only does the tertiary base physostigmine displace the quarternary base acetylcholine but also that acetylcholine displaces physostigmine from cholinesterase (8). Yet, these two agents are of a different molecular structure. They have in common, however a nitrogen with at least three methyl or similar substituting groups. Accordingly, the antagonistic effect of E 4 N against pilocarpine and arecoline, which like physostigmine are tertiary bases, could, dependent on the definition of competition, also be due to competition with these bases.

The unexpected finding that E 4 N antagonizes the effect not only of the bases previously mentioned but also that of potassium obviously raises the question whether there could not be found a unitarian explanation of the antagonism in both cases.

Quarternary ammonium salts are strong electrolytes characterized by the great stability of their cations which closely resemble those of alkali metals (9). Accordingly, the antagonism of E 4 N against potassium could perhaps be interpreted by assuming a competition of the cation E 4 N (10) with the cation potassium. Could such an assumption also explain the antagonism of E 4 N against the effect of the vagomimetic bases? It has been conclusively proven that potassium liberates AcCh (11). As to the question of whether AcCh in its turn liberates potassium, Ing and Wright (9) suggested that AcCh might replace inorganic cations in the structures upon which it acts. In addition there already exists some experimental evidence for the correctness of this view (12) yet not enough by far to prove it beyond a doubt and to show conclusively that the potassium liberated is responsible for the final effect of the bases. If, however, in the future this could be proven beyond a doubt, the conclusion could be drawn that E 4 N does not compete with the bases themselves but with the potassium released by them.

The antagonism of atropine on the other hand exerted against the action of the vagomimetic bases not, however, against that of potassium could be interpreted and in fact has been interpreted as competition with the bases themselves (13). Such a competition would obviously prevent the release of potassium by them and hereby their effect would be absent.

So far we have dealt with a hypothesis concerning the mechanism of the antagonistic action of E 4 N where competition for the same receptor at the effector

cell would be involved. We have, however, to bear in mind also the possibility of a functional antagonism. This means an antagonism emerging not from competition for the receptor but from an opposite functional effect of the antagonizing agents. It is generally assumed that potassium and acetylcholine produce a change of the cell surfaces resulting in a change of their polarized state (14). It cannot be excluded so far that the antagonistic action of E 4 N is due to such an influence opposed to that of the vagomimetic drugs and of potassium respectively.

The hypotheses discussed are not the only ones which could be advanced.

SUMMARY

1. Tetraethylammonium in high concentrations has an action by itself. It increases the contractions of the frog's heart.
2. It antagonizes in concentrations below those augmenting the heart beats, the depressant actions not only of vagomimetic quaternary bases but also those of tertiary bases as pilocarpine and arecoline and of potassium.
3. Possible mechanisms of these antagonisms are discussed.

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THE RELATION OF THE PLASMA CONCENTRATION OF QUINACRINE TO ITS ANTIMALARIAL ACTIVITY¹

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It has been generally assumed in studying the antimalarial action of quinacrine that its antimalarial activity may be related to its concentration in the plasma (1, 2, 3, 4). However, no unequivocal evidence has been presented to show that the antimalarial activity of quinacrine is at all correlated with the variations in plasma concentrations which occur in different individuals on the same daily dosage of drug. In studies on the action of quinacrine in lophurae malaria in the duck direct evidence has been obtained that the same plasma concentration obtained from different doses does not have the same antimalarial effect. In addition, these studies indicate that variations in plasma concentrations obtained in different ducks on the same quinacrine dosage are not correlated with antimalarial activity.

METHODS. White Pekin ducks of different weights (200-3000 gms.) were used depending on the particular information desired from the experiment. *Plasmodium lophurae* was the infecting organism. The general technique of our infections has been described (5, 6). Quinacrine was given as the dihydrochloride, but all figures are expressed as the base. With the exception of one experiment where the drug-diet method (5) was used, the drug was administered as a solution (10 cc. per kilogram) by stomach tube. Samples for analysis were always taken 22 to 24 hours after a dose of quinacrine unless otherwise stated. Duplicate analyses were done on all samples. All blood samples were drawn from the right jugular vein. When blood was drawn without sacrificing the duck, the duplicate samples were taken in separate syringes by separate venipunctures; when the duck was sacrificed, a single syringe was used, but the jugular vein was exposed. Clotting was prevented by potassium oxalate. In the collection and preparation of samples the precautions mentioned by Shannon et al. (1) were observed. The liver, muscle and skin samples were homogenized in 0.05 N hydrochloric acid with a Waring blender.

Quinacrine was determined by the method of Brodie and Udenfriend (7). The ethylene dichloride extracts were in all cases washed with alkali. Fluorescence was determined in the ethylene dichloride extract after making it acid with trichloroacetic acid (8). Determinations made on the tissues of ducks which had received no drug were always zero.

Concentrations of quinacrine in plasma as low as 8 micrograms per liter may be considered significant. Concentrations of 30, 100 and 100 micrograms per kilogram may be considered significant for erythrocytes, muscle and liver respectively.

RESULTS. *Variations in plasma concentrations.* When determinations of the plasma concentration of quinacrine were done in individual ducks fed a drug-diet containing quinacrine, great variations were encountered. It appeared advisable to repeat the experiment with a single-daily oral dose. Ducks having an initial weight of about 200 grams were used in these experiments and were re-

¹ This investigation was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University.

weighed every second day. The results of these two experiments are summarized in table 1. The average daily intake of quinacrine on the drug-diet was 71 milligrams per kilogram and the daily dosage in the tubing experiment was 75 milligrams per kilogram. The range of plasma values obtained as well as the average is given in the table.

In order to determine the variation in the same duck at different times, ducks weighing 1400-1900 grams were given 75 milligrams of quinacrine per kilogram per day. The variations in the concentration of drug in the plasma are shown in table 2.

TABLE 1
*The concentration of quinacrine in the plasma of young ducks**

DAY OF EXPERIMENT	DRUG-DIET		DAILY TUBING	
	Average	Range	Average	Range
	<i>micrograms per liter</i>			
2	41	22-55	75	40-124
4	217	55-670		
5			169	88-247
6	101	49-194		
7			62	52-85
8	606	108-1220		
9			68	62-73
12	430	92-1350		
14			322	90-758
17			386	78-1190
19	231	86-379		
21			117†	99-136
22			392‡	240-668
23			331‡	148-691
24	86	76-105		

* Ducks weighing 400-800 grams at the time of sacrifice were given 75 mg. quinacrine per kilogram by stomach tube or were fed a drug-diet giving approximately the same daily dose of drug. Four ducks were used for each average unless otherwise indicated.

† Two ducks were used.

‡ Three ducks were used.

Plasma and tissue concentrations after repeated doses. The simplest explanation of the wide variations in the concentration of quinacrine in the plasma is that the distribution ratio between plasma and tissues is quite variable in different ducks and in the same duck at different times. To test this hypothesis, determinations of quinacrine were done on tissues of ducks treated daily with a dose of quinacrine for 17 to 33 days. Two lots of ducks weighing 2000 and 600 grams (weight when sacrificed) respectively, were given a dose of 75 milligrams per kilogram daily; another lot, weighing around 2000 grams was given a daily dose of 5 milligrams per kilogram. The results of these experiments are summarized in tables 3, 4 and 5. It is obvious from an examination of the data given in these three tables that there is no correlation between plasma concentrations of quinacrine produced

by the same dose and the concentration of quinacrine found in erythrocytes, liver, muscle or skin. In addition, there is much less variability in the erythrocyte and tissue concentrations than in the plasma concentrations. Erythrocyte and tissue concentrations of quinacrine are lower with the small dose than with the large dose, but the same plasma concentrations obtained with the two doses are accompanied by markedly different erythrocyte and tissue concentrations. It is clear that the wide variations in plasma concentration encountered with the

TABLE 2
*The concentration of quinacrine in the plasma of adult ducks**

DUCK NUMBER	DAY OF EXPERIMENT								
	3	5	7	9	12	15	20	26	27
	<i>micrograms per liter</i>								
19 G	406	280	260	254	542	382	690		487
20 G	246	662	2060	5850	4100	880	286	1220	
26 G	704	1600	1020	460	350	314	230		616
25 G	620	748	602	520	1130	1880			

* Ducks weighing 1400 to 1900 grams were given a daily dose of 75 mg. of quinacrine per kilogram.

TABLE 3
*The concentration of quinacrine in the plasma and tissues of adult ducks given a large daily dose**

MICROGRAMS PER KILOGRAM			
Plasma	Erythrocytes	Liver	Muscle
1170	14,400	2,150,000	289,000
561	10,600	4,390,000	101,000
446	10,100	3,040,000	141,000
370	13,300	2,880,000	140,000
328	14,500	5,860,000	157,000
266	7,140	4,040,000	107,000
214	13,200	5,210,000	138,000
479†	11,900	3,940,000	153,000

* Ducks weighing 1900 to 2200 grams were given 28 to 33 daily doses of 75 mg. of quinacrine per kilogram then were sacrificed.

† Values below the line are the means of the values above the line.

same dosage are due to a variation in distribution ratio of quinacrine between plasma and tissues and it is probable that the variations in plasma concentration do not reflect variations in the total amount of quinacrine in the animal.

Plasma and tissue concentrations after single doses. The fact that tissue and especially erythrocyte concentrations of quinacrine are independent of and less variable than plasma concentrations obtained with a given dosage suggests that antimalarial activity may not be at all correlated with plasma concentrations of

quinacrine. It is obvious that if wide variations in plasma concentrations occurred after single doses of quinacrine, it would be possible to determine the relation of various plasma concentrations to the concentration of quinacrine in parasitized erythrocytes and to the effect of the drug on parasitemia. Both of

TABLE 4

*The concentration of quinacrine in the plasma and tissues of young ducks given a large daily dose**

MICROGRAMS PER KILOGRAM			
Plasma	Skin	Liver	Muscle
1188	78,900	2,030,000	36,400
691		2,580,000	42,200
668		2,880,000	97,200
269		3,090,000	68,000
240		1,140,000	31,000
186	118,000	3,230,000	141,000
154		3,100,000	67,200
148		3,730,000	49,800
136	127,000	2,940,000	108,000
99	106,000	1,950,000	96,300
378†	108,000	2,670,000	73,700

* Ducks were given 75 mg. of quinacrine per kilogram daily for 17 to 23 days. They weighed 400-800 grams at the time of sacrifice.

† Values below the line are the means of the values above the line.

TABLE 5

*The concentration of quinacrine in the plasma and tissues of adult ducks given a small daily dose**

MICROGRAMS PER KILOGRAM			
Plasma	Erythrocytes	Liver	Muscle
49	948	1,160,000	4,440
65	2,050	1,010,000	9,060
125	1,710	1,330,000	3,750
242	1,890	1,900,000	3,850
290	1,550	1,300,000	5,880
154†	1,630	1,340,000	5,400

* Ducks weighing 1700 to 2500 grams were given 29 to 33 daily doses of 5 mg. of quinacrine per kilogram then were sacrificed.

† Values below the line are the means of the values above the line.

these objectives were attained by experiments done on normal and infected ducks given single doses of quinacrine.

The results of the administration of single doses of 75 and 5 milligrams of quinacrine per kilogram are summarized in tables 6 and 7. Just as great a

variation in plasma concentrations is found after one dose as after repeated doses. The concentrations in erythrocytes and tissues are less variable than those in plasma, and there is no correlation between the tissue concentrations and the plasma concentrations. As is to be expected, the erythrocyte and tissue concen-

TABLE 6

*The concentration of quinacrine in the plasma and tissues of adult ducks given a single large dose**

MICROGRAMS PER KILOGRAM				
Plasma	Erythrocytes	Leucocytes	Liver	Muscle
104	1,030		420,000	6,930
141	1,230		618,000	7,920
178	1,090	15,700	289,000	
254	1,700	15,800	319,000	
270	807		665,000	9,470
308	1,930	39,300	433,000	
513	1,300		1,200,000	9,020
587	1,480		1,060,000	5,800
820	1,560	19,600	337,000	
353†	1,350	22,600	593,000	7,830

* Ducks weighing 1800 to 2600 grams were given a single dose of 75 mg. of quinacrine per kilogram and were sacrificed 24 hours later.

† Values below the line are the means of the values above the line.

TABLE 7

*The concentration of quinacrine in the plasma and tissues of adult ducks given a small dose**

MICROGRAMS PER KILOGRAM		
PLASMA	ERYTHROCYTES	LIVER
98	410	52,400
72	294	69,600
51	130	79,900
38	283	73,900
28	264	66,700
28	142	72,500
4	162	61,000
46†	241	68,000

* Ducks weighing 1700 to 2400 grams were given a single dose of 5 mg. of quinacrine per kilogram and were sacrificed 24 hours later.

† Values below the line are the means of the values above the line.

trations are much lower following a single dose than they are following repeated doses. However, the range of the plasma concentrations obtained from the single dose overlaps the range obtained from 28 daily doses.

Plasma and parasitized erythrocyte concentrations. In order to determine the

concentration of quinacrine in parasitized erythrocytes, ducks were infected with *P. lophurae*. When about 30 per cent of the erythrocytes were parasitized a single dose of quinacrine was given. Twenty-four hours later the animals were sacrificed, plasma and erythrocytes were obtained for analysis, and the per cent of erythrocytes parasitized was determined. In the case of the larger dose, many parasites were obviously dead since they no longer contained chromatin material; in the case of the small dose, no indication was obtained that any large proportion of the parasites were dead. From the concentration of quinacrine in the erythrocytes of uninfected ducks (tables 6 and 7), the concentration in the erythrocytes of the parasitized ducks, and the per cent of erythrocytes parasitized, the concentration of quinacrine per kilogram of parasitized erythrocytes could be calculated. The total per cent of erythrocytes parasit-

TABLE 8

*The concentration of quinacrine in the parasitized erythrocytes of adult ducks**

PER CENT OF ERYTHROCYTES PARASITIZED		MICROGRAMS PER KILOGRAM		
Total	Alive	Plasma	Erythrocytes	Parasitized erythrocytes (calculated)
33	19	126	5,880	15,100
29	18	228	8,720	26,800
26	16	238	4,070	11,800
47	25	242	11,200	22,300
31	19	318	6,780	18,900
37	19	332	5,120	11,500
20	10	336	1,050	
30	17	382	7,720	22,600
32	21	581	7,230	19,800
		309†	6,419	18,600

* Ducks infected with *P. lophurae* and weighing 1600 to 2800 grams were given a single dose of 75 mg. of quinacrine per kilogram and were sacrificed 24 hours later.

† Values below the line are the means of the values above the line.

ized rather than the per cent of erythrocytes containing living parasites was used for this calculation.

The results are given in tables 8 and 9. It is obvious from an examination of the data that there is no correlation between plasma concentration and the concentration in parasitized erythrocytes, and there is less variability in the concentration in parasitized erythrocytes. A comparison of the average concentration in normal erythrocytes (1350 and 241 for the large and small doses respectively) with the average concentration in parasitized erythrocytes (18600 and 12700 for the large and small doses respectively) suggests that there is extensive localization of quinacrine in the parasite. However, this is apparently not proportional to either dosage or plasma level for a fifteen-fold increase in dosage or a five-fold increase in mean plasma level results in only about a fifty per cent increase in the concentration of drug in the parasitized erythrocytes.

It would appear from a consideration of the above data that the plasma concentrations found following a given dosage are not related to antimalarial activity since there is no correlation between plasma concentration and concentration of quinacrine in the parasites. This is based on the premise that the concentration of quinacrine in the parasites determines antimalarial activity.

Relation of therapeutic effect to plasma concentration and dosage. A direct experiment was performed to investigate the relation of antimalarial activity to plasma concentration and dosage. Ducks weighing about 200 grams were infected with *P. lophurae*. On the third day after infection, various doses of quinacrine were administered. Ducks were sacrificed for determinations of the quinacrine concentration in the plasma twenty-four, forty-eight and seventy-two hours later. The height of the parasitemia was determined on the third, fourth, fifth and sixth days after infection in all ducks still alive. The per cent of erythrocytes parasitized in untreated animals averaged 10, 16, 44 and 65 on the

TABLE 9

*The concentration of quinacrine in the parasitized erythrocytes of adult ducks**

PER CENT OF ERYTHROCYTES PARASITIZED		MICROGRAMS PER KILOGRAM		
Total	Alive	Plasma	Erythrocytes	Parasitized erythrocytes (calculated)
29	29	12	4,300	14,200
28	28	44	2,520	8,400
35	35	60	4,570	12,600
26	20	88	2,440	8,700
24	23	94	4,360	17,400
30	28	110	4,710	15,100
		68†	3,820	12,700

* Ducks infected with *P. lophurae* and weighing 1600 to 2600 grams were given a single dose of 5 mg. of quinacrine per kilogram and were sacrificed 24 hours later.

† Values below the line are the means of the values about the line.

third, fourth, fifth and sixth day respectively. In all 120 ducks were used, an experiment of 30 being performed at one time with three doses of quinacrine.

The antimalarial activity was assessed as follows: no effect (0) where the percentage of erythrocytes parasitized was greater than fifty per cent of controls, partial suppression of parasitemia (+) where the percentage of erythrocytes parasitized was less than fifty per cent of the control value but greater than one parasitized cell per thousand, complete suppression (●) where the parasitemia was less than one parasitized erythrocyte per thousand.

The results are given in figure 1, where the twenty-four, forty-eight and seventy-two hour values are plotted separately. There appears to be very little if any correlation between plasma concentration and therapeutic effect, but the correlation between dosage and therapeutic effect is good.

Disappearance of quinacrine from plasma and tissues after a single dose. Thirteen ducks were given a single dose of 75 milligrams of quinacrine per kilogram.

Three were bled at various times and the plasma concentration of quinacrine determined; they were sacrificed fourteen days after the drug was given, and the quinacrine concentration was determined in plasma, erythrocytes, liver and muscle (table 10). Five were sacrificed 14 days and five were sacrificed 28 days after dosage (table 11). These data show that quinacrine (or at least total acridines) disappeared much more slowly from the tissues than from the plasma. In fact, two weeks after the dose of quinacrine the plasma level is at most only two per cent of that found after twenty-four hours, while the red cell and muscle

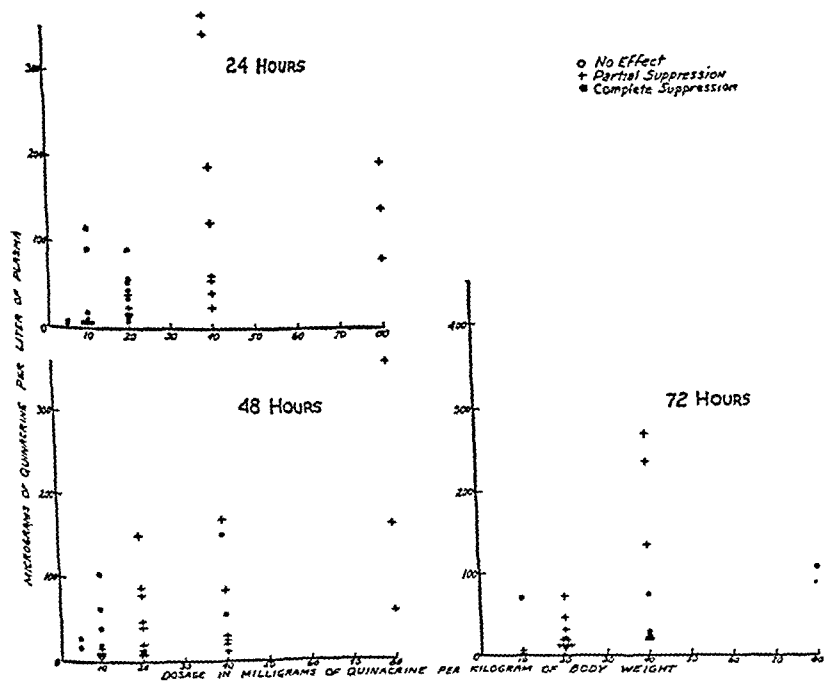


FIG. 1. THE RELATIONSHIP OF PLASMA CONCENTRATION, DOSAGE, AND ANTI-MALARIAL EFFECT OF QUINACRINE IN *LOPHURAE* MALARIA IN THE DUCK

concentrations are about ten per cent and the liver concentration about fifty per cent of that found after twenty-four hours (tables 6 and 10). Four weeks after a single dose, when at most only traces are present in plasma, the muscle concentration is about three per cent and the liver concentration over ten per cent of the concentrations present at twenty-four hours (tables 6 and 11). This, of course, means that the tissue-plasma ratio is increased during the disappearance of quinacrine from the organism.

DISCUSSION. The data obtained in this investigation show that the plasma concentration of quinacrine does not necessarily indicate the concentration of

the drug in the tissues of the duck. Variations in plasma concentration in different ducks or in the same duck at different times, where quinacrine intake is constant, are not due entirely to differences in absorption, excretion and/or degradation, but are due mainly to variations in the distribution ratio of quina-

TABLE 10

*The concentration of quinacrine in the plasma and tissues of adult ducks 14 days after a single dose**

MICROGRAMS PER KILOGRAM			
Plasma	Erythrocytes	Liver	Muscle
0	82	234,000	186
0	80	214,000	450
0	170	357,000	1150
0	157	166,000	303
0	214	246,000	650
20†	95	245,000	386
16†	278	52,700	1630
0†	175	277,000	526
4†	156	224,000	660

* Ducks weighing 2000 to 2800 grams were given a single dose of 75 mg. of quinacrine per kilogram and were sacrificed 14 days later.

† Values below the line are the means of values above the line.

‡ Blood samples for analysis were taken from these ducks 4 to 6 times during the 14 day period.

TABLE 11

*The concentration of quinacrine in the plasma and tissues of adult ducks 28 days after a single dose**

MICROGRAMS PER KILOGRAM			
Plasma	Erythrocytes	Liver	Muscle
0	36	116,000	340
0	34	24,900	203
0	60	152,000	177
0	40	88,400	262
0	0	50,700	144
0†	34	86,400	225

* Ducks weighing 2200 to 3000 grams were given a single dose of 75 mg. of quinacrine per kilogram and were sacrificed 28 days later.

† Values below the line are the means of the values above the line.

crine between plasma and tissues. Quinacrine is quite different from the sulfonamides or quinine. Sulfonamides are not concentrated in the tissues while quinine is concentrated to only a slight extent. Quinacrine may be present in tissues in concentrations several thousand times that present in plasma.

The fact that the variations in plasma concentration from any given dosage of quinacrine are not correlated with the concentration of drug in erythrocytes or in the parasites suggests strongly that whole plasma concentration is not correlated with antimalarial activity. Direct therapeutic experiments support this thesis for lophurae malaria in the duck. Here it is quite clear that antimalarial activity is well correlated with the dosage of quinacrine but very poorly or not at all correlated with plasma concentrations. Variations in plasma concentrations obtained by varying the dosage appear to have an entirely different significance from variations in plasma concentrations occurring with the same dosage.

The distribution of quinacrine between plasma and tissues is quite complex. When one considers the fact that there is several thousand times the concentration of the drug in tissues that there is in the plasma, and that this extensive localization may be the resultant of many factors, it is doubtful if a simple explanation will be found for the marked variations in the tissue-plasma distribution ratio.

The results reported in this communication were all obtained in the duck. The question immediately arises as to whether conclusions drawn from these experiments can be transferred to man. While no unequivocal answer can be given, it appears that in human malaria the situation may not be different and that here antimalarial activity may not be closely correlated with the plasma concentration of quinacrine. An examination of published data would lead us to believe that the supposed relationship of plasma concentration of quinacrine to antimalarial activity is based mainly on analogy to the sulfonamides in bacterial infections and quinine in malaria. Certainly no unequivocal evidence has been given that the plasma concentration of quinacrine in man is related to antimalarial activity.

Our reasons for believing that it is very probable that the situation in regard to quinacrine and lophurae malaria in the duck is very similar to that obtaining in man and human malaria may be briefly stated. One might argue that in the duck as opposed to man, one is determining mainly degradation products and not quinacrine. An examination was made by the counter-current distribution method (9) of the ethylene dichloride extracts from plasmas taken from three ducks twenty-four hours after a single dose of quinacrine.² This indicated that about ninety per cent of the fluorescent material present was quinacrine. This is in good agreement with the findings in human plasma after administration of quinacrine (7). Qualitatively, the same lack of correlation between plasma and tissue concentrations in the duck is seen twenty-four hours after a single dose of quinacrine as occurs twenty-four hours after thirty daily doses. These two considerations make it unlikely that the quinacrine method is much less specific for the duck than for the human.

However, it should be clearly understood that the specificity of the quinacrine method has been shown only for plasma taken from ducks twenty-four hours after a single dose. It is quite possible that after a longer period plasma may contain

² We are greatly indebted to Dr. B. B. Brodie of New York University Unit of the Goldwater Memorial Hospital for making this analysis of homogeneity.

more ethylene dichloride extractable fluorescent degradation products. In the case of liver, there is reason to believe that degradation products are present, and determined with quinacrine. Measurement of the concentration of quinacrine in the ethylene dichloride extract of liver by means of fluorescence and by transmission at 425 $m\mu$ revealed a slight difference. Determination of the absorption spectra of these extracts indicates that a large part of colored material consist of acridines.³

An examination of published records would appear to indicate that variations in plasma levels and plasma-tissue distribution ratios seen in the duck are qualitatively similar to what occurs in man, although the variations may be more pronounced in the duck than in man. Thus, in the most careful and controlled study on quinacrine (10), we find variations of 300 to 400 per cent in plasma level in the same individual at different times and as great a variation as is seen in ducks in different individuals. In the human, practically all our knowledge of the physiological disposition of quinacrine has been obtained from plasma concentration studies; of necessity, no tissue analyses have been performed in man. One does not find a study in either man or any mammal of the disappearance of quinacrine from the organism. The data of the Fort Knox Study (10) would indicate that practically all quinacrine should be removed from the body in four or five weeks as judged by plasma concentrations. However, early studies (11) indicate that fluorescent material can be found in the urine seventy days after cessation of administration. This reminds one of the disappearance from the duck.

In man, deductions in regard to the pharmacology of quinacrine have been made mainly from determination of plasma concentrations. The assumption has been made that the plasma concentration is correlated with the concentrations of quinacrine in tissues and in parasites. This assumption is not true for the duck; no evidence has been presented that it is true for man. If one considers the wide variations in plasma concentration encountered in different individuals on a dosage of 0.6 gram of quinacrine (10), it is difficult to explain on the theory of the correlation of plasma concentration with antimalarial activity, the complete suppression of malaria in all individuals (12).

³ After our manuscript was sent in for publication, Dr. John V. Taggart of the Third (New York University) Medical Division, Goldwater Memorial Hospital examined by the counter-current distribution method the ethylene dichloride extractable fluorescent material from specimens of the livers of ducks used in our experiments. He kindly allows us to give the following information. In the liver from a duck sacrificed 24 hours after a single dose of 75 mg. quinacrine base per kg. 90 per cent of the total fluorescence was due to quinacrine while 10 per cent was due to degradation products; in pooled livers from two ducks sacrificed 14 days after a single dose of 75 mg. per kg. only 15 per cent of the total fluorescence was due to quinacrine while 85 per cent was due to degradation products, and in the liver of a duck sacrificed 24 hours after the last of 27 daily doses of 75 mg. per kg. the quinacrine fluorescence represented 65 per cent of the total while only 35 per cent could be ascribed to degradation products. These data and similar data from mammals will be published shortly by Dr. Taggart.

SUMMARY

1. Marked variations in the plasma concentration of quinacrine occur in ducks maintained on a constant daily dosage. This variation occurs in individual ducks and in the same duck on different days.

2. There is no correlation between the plasma concentration of quinacrine and its concentration in erythrocytes and tissues either after repeated daily doses or after a single dose. The erythrocyte and tissue concentrations are less variable than the plasma concentrations.

3. Quinacrine is localized in the parasitized erythrocyte. The concentration in the parasitized erythrocytes is less variable than and not correlated with plasma concentrations.

4. Direct experiment indicates that for lophurae malaria in the duck, there is little or no correlation between plasma concentration and therapeutic effect, but good correlation between dosage and therapeutic effect.

5. After administration of a single dose to the duck quinacrine (or total acridines) disappears from the plasma much faster than from the liver. The rate of disappearance of the drug from muscle and erythrocytes is intermediate to that for plasma and liver.

6. The behavior of quinacrine in the duck and the absence of unequivocal evidence to the contrary in man lead us to believe that the situation in man is very similar to that in the duck.

We wish to thank Eleanor R. Mann, Charlotte Kennedy, Jean Hunt and Marjorie L. McBurney for technical assistance.

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STUDIES ON THE PHARMACOLOGY AND ACUTE TOXICITY OF COMPOUNDS WITH MARIHUANA ACTIVITY¹

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The various data on the toxicity of marihuana (13, 8) refer to crude hemp preparations. They are of limited interest and not necessarily correlated with the toxicity of the active principles of the drug. The first prerequisite for a systematic study of the toxic aspects of the pure principles is their isolation and identification. This was accomplished several years ago (1, 8, 14).

Whereas the specific activity of more than seventy pure substances with marihuana activity has been studied quantitatively during the past seven years (8, 5, 6), only four have become available in amounts sufficient to permit investigation of their general toxicity. Three of these compounds were the main components of hemp oil. Two of them, a natural tetrahydrocannabinol and cannabinol, possess marihuana activity, whereas the third, cannabidiol, is inert. The fourth, 1-hydroxy-3-*n*-hexyl-6,6,9-trimethyl-7,8,9,10-tetrahydro-6-dibenzopyran (parahexyl), a marihuana-active congener of tetrahydrocannabinol, is a synthetic compound and was chosen for comparison with the natural substances.

SUBSTANCES STUDIED. I. *Tetrahydrocannabinol (acetate; THC)*. The compound employed in this study³ was the highly marihuana-active laevorotatory natural tetrahydrocannabinol from Oriental cannabis resin (charas) previously described (14). It was prepared from the resin after conversion into the acetyl ester and was employed only in the ester form.

II. *Cannabinol*. Two different specimens of this component of hemp oil, the feeble marihuana activity of which was only recently demonstrated (9), were employed. The one was a highly purified, repeatedly recrystallized cannabinol with an MP of 75.5–76.0°C., prepared from charas (7),³ the other one was a less purified synthetic product (4).⁴

III. *Cannabidiol*. This was available as the pure laevorotatory substance prepared from American hemp oil (2).⁴

IV. *Parahexyl*. This compound was the racemic synthetic hexyl homolog of an isomer of tetrahydrocannabinol, as first synthesized by R. Adams (3).⁵

TECHNIC OF ADMINISTRATION. Experiments with the substances listed above were complicated by their poor water solubility and by the great tolerance of

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⁵ Supplied by the Abbott Laboratories, Inc., Chicago, Illinois, through the courtesy of Dr. R. K. Richards.

all species of animals to those actions with which this study was chiefly concerned.

Organic solvents had to be used. The compounds were all soluble in any proportion in ethanol and, as free alcohols, in propylene glycol. The ester, THC, was administered in dipropylene glycol. The glycol solvents were given preference because of their lower toxicity. The oral L.D.₅₀ of propylene glycol in the strain of mice employed was determined to be 26.0 cc./kg. \pm 0.70, which is in satisfactory accordance with the value of 23.9 cc./kg. reported by others (15), and that of dipropylene glycol to be 20.1 cc./kg. \pm 0.65. In some instances, ethanol had to be employed, the L.D.₅₀ of which is much smaller. In a few experiments with smaller doses, homogenized, aqueous emulsions of concentrated solutions in oil, stabilized by the aid of lecithin, were used.

For oral administration in mice, the stomach tube was employed. In dogs, the undiluted substances were fed in gelatin capsules.

LETHAL DOSE. The data relating to the mean lethal dose of the four substances are summarized in table 1. When possible, the L.D.₅₀ was established by graphic interpolation (12). In these cases, the reliability of the result is illustrated by the standard error. In many instances the number of experiments was too small to allow statistical evaluation of the L.D.₅₀, and only a rough estimate of the threshold lethal range was obtained.

When organic solvents were used, i.e., in all experiments except the oral ones in dogs, their amounts were kept under one-half the L.D.₅₀ whenever possible. In the higher dose experiments the amount of the solvent was sometimes greater than one-half the L.D.₅₀.

The figures obtained should be considered as minimum values of the L.D.₅₀. Participation of the solvent in the lethal effect cannot be completely excluded even in those experiments in which its amount was only a relatively small fraction of the L.D.₅₀.

The time interval between administration and death was usually one or more hours or occasionally even days, with the following exceptions: (a) In all intravenous experiments in mice, death followed the injection within a few minutes. (b) Death was similarly rapid in rabbits when an intravenous dose was administered in 20 per cent and not, as usually, in 80 per cent concentration. In this case, the dose of the solvent was close to, although always less than, 0.7 cc./kg., an amount which was well tolerated when given without solute. (c) When the solvent was ethanol, the survival time was shorter and the lethal dose smaller.

EFFECTS ON CENTRAL NERVOUS SYSTEM. a. *Ataxia.* An extensive literature (11, 8) describes the numerous typical features of the motor disturbances produced by marihuana in the dog, the classic test animal for this drug. The picture of marihuana ataxia in the dog is a complex of astasia, dysbasia and dysmetria, manifested in adiadokokinesia, swaying, staggering and stumbling due to lateral, frontal and caudal pulsion. Also observed are increased muscle tension, coarse twitching, and weakness at rest characterized by dropping of the head and "slipping" of the extremities.

Other species differ from the dog in that a varying number of the components of the characteristic pattern of ataxia is lacking. Motor symptoms in cats, in

TABLE 1
Mean lethal doses of marihuana components and related substances

SPECIES	ROUTE	PARAHXYL			TETRAHYDROCANNABINOL			CANNABINOL		CANNABIDIOL	
		No. of expts.	L.D. ₅₀		No. of expts.	L.D. ₅₀		No. of expts.	L.D. ₅₀ gm./kg.	No. of expts.	L.D. ₅₀ gm./kg.
			gm./kg.	St. error		gm./kg.	St. error				
Mouse	oral	72	13.5	1.85	62	>21.6		3	13.5	11	>12.7
Dog	oral	1	<0.93								
Mouse	subcut.	21	>34.0		23	>11.0					
Mouse	vein	40	0.170	0.050	22	0.175	0.024				
Mouse	vein	8	0.200*								
Rabbit	vein	15	0.143	0.026	8	0.155	0.02	1	<0.126		
Dog	vein	22	0.223	0.041	9	0.100		4	>0.254		
Guinea pig	intraperiton.	7	0.850								

* In aqueous emulsion.

which only the low-dosage range was studied, resemble those in the dog, but somewhat higher doses are required to elicit them. Yet the symptoms of swaying and stumbling are never as manifest as in the dog. This may be partly explained by the fact that the cat rarely assumes an upright posture with legs extended. In rabbits, unequivocal symptoms of ataxia occurred only rarely. In most rabbits only the less characteristic symptoms are present, "slipping" of the extremities and dropping of the head. A frequent feature in rabbits and the dominant one in guinea pigs and mice is hypomotility and motor weakness. When the animal walks, there are often a lack of direction, a tendency to zig-zag and sometimes circling movements. The doses producing motor effects in all three species of rodents are at least twenty to fifty times higher than the threshold dose for ataxia in dogs, and are higher in mice and guinea pigs than in rabbits. No motor symptoms were observed with cannabidiol.

b. *Catalepsy*. Cateleptic phenomena, a great variety of which has been described in the dog (8), were observed in all species studied.

Rabbits, like dogs, maintained an extended position when suspended prone in space by supports under the pelvis and upper thorax.

In the mouse, the cataleptic state is best manifested when the animal is placed prone upon an arrangement (brim of a beaker or two parallel wires) for supporting it only at the thighs and the jaw. After an adequate dose of parahexyl or THC, an animal of otherwise normal agility maintains this extended position for an indefinite time without sagging, until an adequate stimulus arouses it. A similar catalepsy test can be used in guinea pigs.

The cateleptic response is the most sensitive symptom of marihuana action in the mouse. However its usefulness is limited by two circumstances. First: Sub-lethal doses of propylene glycol and dipropylene glycol even in doses as low as 5 cc./kg., can induce a similar position in mice, although it can be distinguished from that seen after marihuana-active substances by sagging and by less responsiveness to stimuli. Secondly, the hypermotility occurring in certain phases of marihuana action interfered with the cateleptic response in a varying percentage of mice. This was particularly true when small doses of ethanol were employed as a solvent.

With cannabinol, a cateleptic response in the mouse was obtained only after a lethal oral dose of 15.4 gm./kg. In the case of cannabidiol the response was absent even after 12.7 gm./kg., the highest dose tested in mice.

c. *Corneal areflexia*. Suppression of the lid reflex of the rabbit is a systemic effect observed after all isomeric tetrahydrocannabinols and numerous congeners. Parahexyl as well as THC were effective even in small doses, but corneal areflexia was not obtained with any dosage of cannabinol and cannabidiol, nor in any species other than the rabbit. A xerotic condition of the cornea was seen in dogs after several days of motor depression produced by near-lethal doses of parahexyl, but the wink reflex was not abolished.

Even in the rabbit, corneal areflexia is by no means a consistent response nor does it parallel other effects of marihuana. When referred to ataxia activity,

the areflexia activity of parahexyl is much greater than that of THC, as was recently reported in detail (10).

d. *Central excitant action.* Whereas states of central excitement and hyperactivity after both parahexyl and THC were rare in dogs and guinea pigs, they were often exhibited by rabbits, and were frequent in mice.

Rabbits, after doses of 100 mg./kg. and up, often showed markedly increased locomotor activity somewhat resembling that produced by apomorphine. Attacks of running, particularly when elicited by a stimulus of touch or pain, would begin with one or several jumps, and precede or interrupt the phases of hypomotility and catalepsy.

In the mouse, motor excitation can be observed with great individual variations and occurs even after very small doses. Attacks of running, jumping and dancing could be elicited by slight stimuli. Simultaneously, the animals were often aggressive and pugnacious. As in the rabbit, excitement and hyperexcitability may prevent cataleptic responses and terminate the cataleptic state.

e. *Hypnotic action.* Typical anesthesia or narcosis was never observed in any species after any dose of the four substances. At the height of the effect of large doses, dogs and sometimes other animals to a lesser extent may exhibit a picture of apathy superficially resembling that produced by a hypnotic. But a characteristic difference from the effects of anesthetics or hypnotics is the ease with which the marihuana animals can be aroused.

GASTRO-INTESTINAL SYMPTOMS. Salivation, retching, nausea and vomiting occur frequently in dogs after oral and intravenous administration of all the marihuana-active substances in doses which produce pronounced ataxia. Such symptoms are unrelated to dosage and are absent in cats.

Diarrhea was observed only in mice. In this species it was a regular symptom in the highest range of oral THC and parahexyl doses when the animals survived more than 15 hours. The only dog succumbing after oral administration of parahexyl developed a profuse bloody diarrhea and had extensive hyperemic areas in the intestinal tract.

RESPIRATORY SYMPTOMS. All dogs succumbing to an intravenous dose of parahexyl exhibited signs of pulmonary edema (11). The weight of the lungs was between two and five times greater than normal.

The respiratory rate was observed in experiments in dogs. An increase did not occur in animals restrained for blood pressure recording, but did occur in unrestrained animals, more frequently after small than after large doses. The increase was transitory, and occurred within 30 minutes after the injection, *i.e.*, before the ataxia was completely developed. The original rate was exceeded by a maximum of between 30 and 100 per cent.

Subsequent to the initial stimulation, the respiration was usually decreased. A decrease was also observed in animals tied to the board for blood pressure recording. The greatest respiratory depression, with no preceding stimulation, was observed in the dog receiving the largest dose of parahexyl (926 mg./kg, by mouth).

There appeared to be no consistent difference in the respiratory symptoms produced by THC and parahexyl.

CIRCULATORY SYMPTOMS. a. *Blood pressure.* No influence upon the blood pressure of dogs was observed in a wide range of dosage of either THC (four experiments) or parahexyl (eight experiments).

b. *Pulse rate.* Increase in pulse rate in man has been repeatedly described as a characteristic action of cannabis preparations, and has also been observed occasionally in the non-anesthetized dog after THC (8). It was observed, however, only in a few out of the 28 experiments in dogs reported here. The only significant rise in pulse rate in an unrestrained animal was seen in one instance after a very large intravenous dose of parahexyl, 326 mg./kg. The increase in this case coincided with the development of labored respiration, and both phenomena may be attributed to a rapidly developing pulmonary edema.

A moderate decrease of the pulse rate was a much more consistent sign. It was always present in unrestrained animals, and was seen in about half of the restrained animals. The decrease appeared not to be related to the size of the dose or to the degree of motor depression. The pulse rate began to decrease soon after the drug was injected and remained slow for hours. The maximum effect usually occurred in the early phase of this period, but in some instances the decrease progressed steadily as long as the animal was observed. The decrease was abolished by sectioning the vagus nerves or by administering atropine.

Pulse irregularities, if present before administration of the drug, occasionally improved after the drug was given, but in dogs with normal sinus rhythm the injection appeared sometimes to produce cardiac irregularities.

DISCUSSION AND CONCLUSIONS. A major conclusion of these studies is that under all conditions the lethal dose of the pure marihuana principles is extremely high compared both with the small doses required for the specific pharmacodynamic effects and with the lethal doses reported for crude hemp preparations. Parahexyl, THC and cannabinal, three congeners of very different marihuana activity, as well as cannabidiol, the main relatively inactive component of hemp oil, can be given to albino mice without persistent harm in larger doses than sodium chloride. The ratio between the oral lethal dose of THC in the mouse and the intravenous threshold dose producing ataxia in the dog is more than 200,000. The ratio between the oral L.D.₅₀ in the mouse and the threshold dose for the psychic action in man is more than 40,000 for THC, and more than 4,500 for parahexyl.

Various findings indicate that the cause of death is not correlated with the mechanism of the specific marihuana action. THC is about six times more effective than parahexyl in producing ataxia in dogs, but has by mouth a greater, and by vein the same L.D.₅₀. The enormous species differences in specific marihuana activity were not paralleled by differences in lethal toxicity. In the mouse, which is refractory to marihuana action, the intravenous L.D.₅₀ is the same as in the dog which is highly susceptible to marihuana-induced ataxia. In the dog, the difference between the loci of the lethal action and the specific

central nervous effect was particularly evident. In this species, death was due to acute intestinal effects after oral, and acute pulmonary alterations after intravenous administration.

The extremely poor water-solubility of the cannabinal and cannabidiol drugs appears not to be the main factor responsible for the low toxicity. Whereas the lethal dose in the mouse may be about 100 times higher by mouth than by vein, the ataxia dose in the dog is only ten to twenty times higher by vein than by mouth, and the lethal dose less than four times higher. In addition to solubility, the sensitivity of different sites of action in different species appears to be a factor of major importance for the nature and the degree of toxicity.

The species differences in sensitivity are such that cataleptic effects were the only manifestation of marihuana action common to all the species of animals studied. Certain actions upon the circulation and respiration, which in the past have been considered to parallel marihuana activity, particularly an increase in pulse rate, were not found to occur with any regularity, nor were they found to be correlated with dosage or specific action. The only regular circulatory change in dogs was a moderate decrease in pulse rate.

An important result of the study of pure hemp substances is the refutation of the general belief in their hypnotic action. Neither the two highly marihuana-active substances nor the two major components of hemp possessing negligible marihuana activity manifested any hypnotic or sedative activity in any dose or species of animals. The actions of marihuana-active compounds upon consciousness, psychic functions and sensori-motor reactions are much more selective. It does not appear justified to align these substances with the hypnotics or anesthetics.

SUMMARY

1. The acute toxicity of the highly marihuana-active natural charas tetrahydrocannabinol (acetate), its somewhat less active synthetic homolog, parahexyl, the feebly active hemp component cannabinal, and the inactive hemp component cannabidiol was studied in mice, rabbits, guinea pigs, cats and dogs.

2. The L.D.₅₀ of all four substances was very high as compared with the dose causing specific symptoms, namely, over 10.0 gm./kg. in mice by mouth and still higher subcutaneously, and between 100 and 230 mg./kg. by vein in mice, rabbits and dogs.

3. The ataxia activity varies widely in different species of animals. Corneal areflexia is elicited only in rabbits. The only sign of marihuana action common to all species of animals is a cataleptic effect. The only consistent circulatory effect in dogs was a moderate decrease in pulse rate. Excessive oral doses in mice produce diarrhea.

4. Hypnotic action is completely absent in all four substances in all species.

5. Lethal effects and specific pharmacologic activity are not correlated. The four substances vary greatly in ataxia potency, but little in lethal dose. Dogs die from pulmonary edema after intravenous administration. One dog succumbed from intestinal hemorrhage following parahexyl given by mouth.

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THE CORRELATION BETWEEN DISTRIBUTION OF ANTIMONY IN TISSUES AND CHEMOTHERAPEUTIC EFFECT IN EXPERIMENTAL LEISHMANIASIS¹

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Until some 30 years ago, visceral leishmaniasis was an incurable disease which, depending upon the locality, carried a mortality rate of 80-96 per cent (1). Following the introduction of tartar emetic by Vianna for the treatment of South American leishmaniasis, De Cristina and Caronia (2) used this tervalent organic antimonial as a therapeutic agent against infantile kala-azar and in the following year Rogers (3) and Muir (4) used the drug for the treatment of leishmaniasis in India. The effect of the chemotherapy on the death rate from this disease was dramatic; however, a prolonged treatment course with tartar emetic was found to be necessary to control the infection, toxic reactions to the drug were frequent, and a considerable number of cases was found to be refractory to the treatment. In 1916 Caronia (5) reported the successful treatment of Mediterranean leishmaniasis with the quinquevalent antimony compound p-acetylaminophenylstibonate (stibacetin or stibinyl). After this a number of quinquevalent antimonials and tervalent antimony compounds were investigated for their therapeutic activity in leishmaniasis (6). The conclusion to be drawn from both clinical and experimental chemotherapeutic investigations is that quinquevalent antimonials are more effective than tervalent compounds in the treatment of leishmaniasis and are also less toxic.

The explanation for the difference in therapeutic effectiveness between the Sb^{III} and Sb^V compounds is not known. The investigation to be reported was undertaken in order to determine whether there is any correlation between the antimony concentration in organs which are heavily parasitized with leishmania in the untreated disease and the therapeutic activity of the drug. Eight tervalent antimony compounds and 7 quinquevalent antimonials were investigated.

MATERIALS AND METHODS. A routine method for testing chemotherapeutic agents in experimental leishmaniasis has been described in detail elsewhere (7). In brief, the method employs 8 week old hamsters (*Cricetulus auratus*) which are injected intraperitoneally with a splenic emulsion obtained from donor hamsters heavily infected with *Leishmania donovani* (Khartoum strain). After about 4 weeks, puncture of the liver usually reveals a heavy uniform infection. The infected hamsters are divided into groups of 5 at random. In the first part of this investigation 5 of these groups were segregated, of which 4 received drugs and 1 was the untreated control. Initially, 2 tervalent antimony compounds, potassium antimony tartrate (tartar emetic) and lithium antimony thiomalate (Anthiomaline) and 2

¹ This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

quinquevalent antimonials, diethylamino salt of sodium antimony gluconate (Stibanose) and the antimony complex of p-aminophenylstibonic acid, p-acetylamino phenylstibonic acid, antimonie acid and diethylamine (Neostibosan) were studied. Subsequently, additional Sb^{III} and Sb^{V} compounds were investigated. The chemical names and dosages employed will be detailed in the section describing results.

The rhodamine-B method for the determination of antimony in biological material (8) was utilized in this investigation. This has been described in detail and evaluated elsewhere (9). The optimal range of the method is 2.0 to 15.0 micrograms of antimony and within this range the standard deviation of the determinations is 0.4 microgram Sb.

RESULTS. The results of the antimony determinations following the administration of tartar emetic, Anthiomaline, Stibanose and Neostibosan are summarized in table 1 together with the total amount of antimony injected in each instance. All the values are expressed as averages and the standard deviation (S.D.) has been calculated in order to describe the range and the distribution of the individual values about the average. It can be seen from table 1 that the total amount of antimony injected is considerably greater in the experiments

TABLE 1

*Antimony content of liver and spleen of hamsters infected with *L. donovani* and treated with four organic antimonials for six days*

(Five hamsters in each group)

DRUG	TOTAL Sb INJECTED	Sb IN LIVER	S D.	TOTAL Sb IN LIVER	S D.	Sb IN SPLEEN	S D	TOTAL Sb IN SPLEEN	S D
	mgm.	$\mu\text{gm/gm}$		mgm.		$\mu\text{gm/gm}$		mgm.	
Tartar Emetic .	2.0	62	20	0.31	0.06	0.9	0.3	Trace	
Anthiomaline	5.3	79	15	0.45	0.08	2.6	1.2	0.001	0.001
Stibanose .	86.0	63	15	0.38	0.07	83.0	30	0.04	0.003
Neostibosan .	22.8	245	27	1.34	0.21	130.0	21	0.04	0.012

with Stibanose and Neostibosan than in those instances in which tartar emetic and Anthiomaline have been administered. Although there is considerable variation in the antimony content of these drugs (e.g. Neostibosan 40% Sb; Anthiomaline, (solution of 1% organic Sb), the chief reason for the discrepancy between the total antimony injected in the trivalent and quinquevalent antimonials is the greater toxicity of the Sb^{III} compounds. In an investigation of the distribution of antimony in chronic toxicity experiments, it was found that the daily doses employed in the experiments of this report were well tolerated in hamsters, whereas any considerable increase in the dosage resulted in weight loss and death within ten days (10).

With the exception of Neostibosan, the concentration and total amount of antimony in the livers of the animals treated with the various drugs are surprisingly uniform. This is due to the greater cumulation of antimony in the liver of the trivalent antimonials as compared to the cumulation of Stibanose. Thus, the percentage of the total antimony dose which is localized in the liver with the four drugs is: Stibanose, 0.4%; Neostibosan, 5.9%; tartar emetic, 15.4%;

Anthiomaline, 8.5%. As can be seen from the standard deviations, there is no significant difference between the concentrations and total amounts of antimony in the liver of animals receiving Stibanose, tartar emetic and Anthiomaline.

The concentration and total amount of antimony in the spleen is very significantly different between the quinquevalent and tervalent antimony compounds. This is explicable on the basis of the smaller total amount of antimony injected in the experiments with the tervalent antimony compounds. It has been found

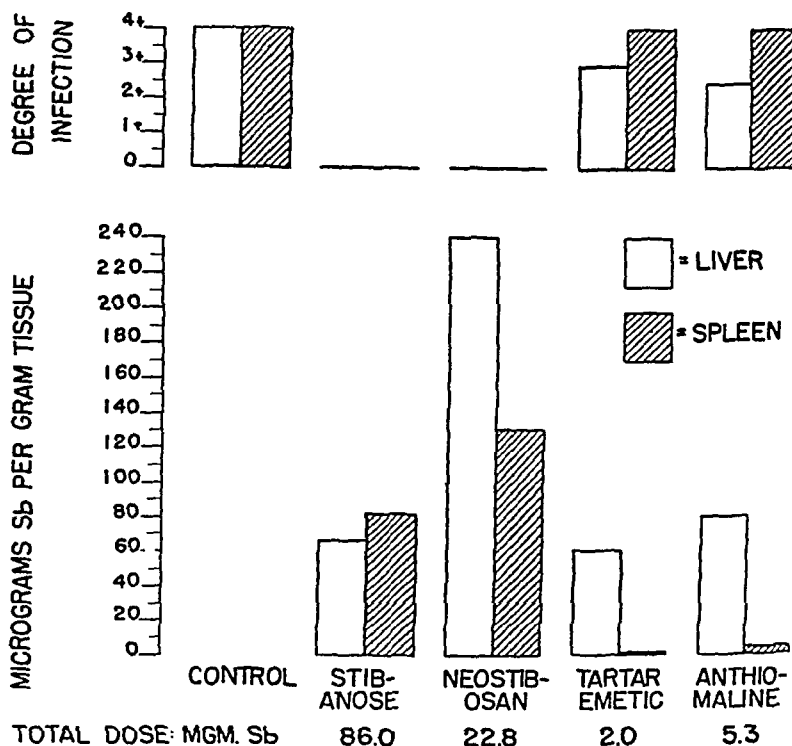


FIG. 1. COMPARISON OF THE ANTIMONY CONTENT OF LIVER AND SPLEEN AND CHEMOTHERAPEUTIC EFFECT AFTER ADMINISTRATION OF 2 TERVALENT AND 2 QUINQUEVALENT ANTIMONIALS

that even after prolonged injection of tartar emetic and Anthiomaline in chronic toxicity experiments, the concentration of antimony in the spleen does not exceed, on the average, 15 μ gm. of antimony per gram (10). As in the observations of the antimony content of the liver, the Sb^V of Neostibosan localized to a greater extent in the spleen than the antimony of Stibanose relative to the total dosage of antimony administered and also in absolute amounts.

Figure 1 correlates the concentration of antimony in the liver and spleen in

the experiments with each drug with the chemotherapeutic results. In every instance the liver and spleen imprints of the untreated controls showed extensive parasitization by the leishmania. These were graded 4+ and served as the basis of comparison in the animals treated with the four drugs. In the experiments with Stibanose and Neostibosan no leishmania could be found in the imprints of the liver and spleen. With tartar emetic and Anthiomaline, the splenic imprints showed the same degree of parasitization as the untreated controls. The liver imprints showed a reduced number of parasites when compared with the controls; however, it is to be emphasized that the method of evaluation of the degree of infection is qualitative and therefore it is not permissible to state unequivocally

TABLE 2

Antimony content of liver and spleen of hamsters infected with L. donovani and treated with various trivalent and quinquevalent antimonials for six days

(Five hamsters in each group.)

DRUG* NO.	CHEMICAL NAME	VAL- ENCE OF Sb	TOTAL Sb IN- JECTED	Sb IN LIVER	S.D.	TOTAL Sb IN LIVER	S.D.	Sb IN SPLEEN	S.D.	TOTAL Sb IN SPLEEN	S.D.
			mgm.	μgm. /gm.		mgm.		μgm. /gm.		mgm.	
6	Antimonythioimidazo- line	III	1.3	40.0	5.0	0.23	0.03	2.0	0.6	0.002	0.0004
8	Trisodium antimony mercaptovalerate	III	3.0	44.0	5.0	0.25	0.02	4.0	1.0	0.0015	0.0005
9	Antimony mercapto- thiazoline	III	1.2	23.0	6.0	0.14	0.04	0.7	0.02	0.0004	0.00001
14	Antimonyl bis (catechol disodium sulfonate)	III	12.4	42.0	19.0	0.18	0.06	10.0	2.7	0.003	0.0002
15	p-carbamidophenyl- antimonyl bis (cate- chol di-sodium sul- fonate)	III	9.6	35.0	12.0	0.20	0.06	5.0	1.0	0.003	0.001
24	Sodium-p (sodiumsulfo- namido) phenyl sti- binyngluconate	III	1.3	10.0	0.4	0.06	0.01	2.0	0.2	0.001	0.0001
17	Sodium antimonyl thio- sorbitol	V	13.6	43.0	13.0	0.29	0.11	6.0	0.2	0.003	0.0006
	Stibanose	V	72.0	54.0	17.0	0.31	0.14	86.0	43.0	0.02	0.003
	Neostibosan	V	26.0	194.0	51.0	1.23	0.27	89.0	13.0	0.05	0.02
20	p-acetyl-aminophenyl stibonate (Stibacce- tin)	V	17.1	74.0	9.0	0.44	0.05	226.0	99.0	0.08	0.04

TABLE 2—*Concluded*

DRUG* NO.	CHEMICAL NAME	VAL- ENCE OF Sb	TOTAL Sb IN- JECTED	Sb IN LIVER	S.D.	TOTAL Sb IN LIVER	S.D.	Sb IN SPLEEN.	S.D.	TOTAL Sb IN SPLEEN	S.D.
			mgm.	μgm. /gm.		mgm.		μgm. /gm.		mgm.	
21	N-glucoside of stibany- lic acid (Neostam)	V	26.0	152.0	21.0	0.77	0.21	115.0	71.0	0.03	0.02
22	Sodium-4-acetyl-amino- phenylstibonyl gluco- nate	V	24.3	56.0	17.0	0.24	0.06	5.0	3.0	0.002	0.0006
25	Sodium p-sulfonamido- phenylstibonyl glu- conate	V	14.9	42.0	8.0	0.22	0.07	2.0	0.6	0.001	0.0004

* The drugs, furnished through the Chemotherapy Center of the National Research Council, were supplied by the following manufacturers:

Nos. 6, 9 and 17: Abbott Laboratories.

No. 8: Wallace Laboratories.

Nos 14, 15, 24, Stibanose, Neostibosan, 22 and 25: Winthrop Chemical Company.

Nos. 20 and 21: Eli Lilly Co. (Neostam is the name used by Burroughs, Wellcome Co. for No. 21.)

Nos. 14 and 15 were diethylaminoethanol salts.

that there is a significant reduction in the total number of parasites in the animals treated with tartar emetic or Anthiomaline.

Following these experiments additional antimony compounds were studied in exactly the same way as has just been described. Table 2, figures 2 and 3, summarize the results on the new compounds investigated. Stibanose and Neostibosan were tested again to serve as a basis of comparison with other quinquevalent antimonials. Inspection of table 2 shows the marked difference in the total amount of antimony usually injected in the experiments with the tervalent antimonials as compared with the quinquevalent antimony compounds which is a reflection of the greater toxicity of the former drugs over the latter. In spite of the low Sb^{III} antimony dosage, the concentration of antimony in the liver of animals receiving the tervalent antimonials is appreciable; in fact, the administration of drug Nos. 6, 8, 14, and 15 produces a liver Sb concentration which is not statistically significantly different from that observed in the experiments with Stibanose. The splenic antimony concentration is consistently low in all of the experiments with tervalent antimonials and also in the experiments with the quinquevalent antimony drugs Nos. 17, 22, and 25. That the low antimony concentration in the spleen observed in these animals can not be explained solely on the basis of the total Sb administered is shown by the results with the quinquevalent Sb compound No. 20 in which the total dose of antimony administered was not very much larger than that of drugs No. 17 and 25, and yet the splenic Sb concentration was higher than in any other experiment. Drug No. 22 was administered in a considerably higher dose than drug No. 20 but led to the deposition of only a small amount of antimony in the spleen.

Figures 2 and 3 present the correlation between the antimony concentration in the liver and spleen and the chemotherapeutic effect of tervalent and quinquevalent antimonials.

DISCUSSION. In both the hamster and man (11) the chief site of localization of leishmania is in the reticulo-endothelial cells of the liver, spleen, bone marrow, and also in abdominal lymph nodes. It is extremely difficult to demonstrate the parasites in the peripheral blood and at autopsy only occasionally can small numbers of leishmania be found in endothelial phagocytes in the interstitial tissues of the suprarenals, thyroid, heart, testes, pancreas, lungs, and prostate. The uneven distribution of leishmania made it impractical to evaluate the chemotherapeutic results in tissues other than those which are highly parasitized and

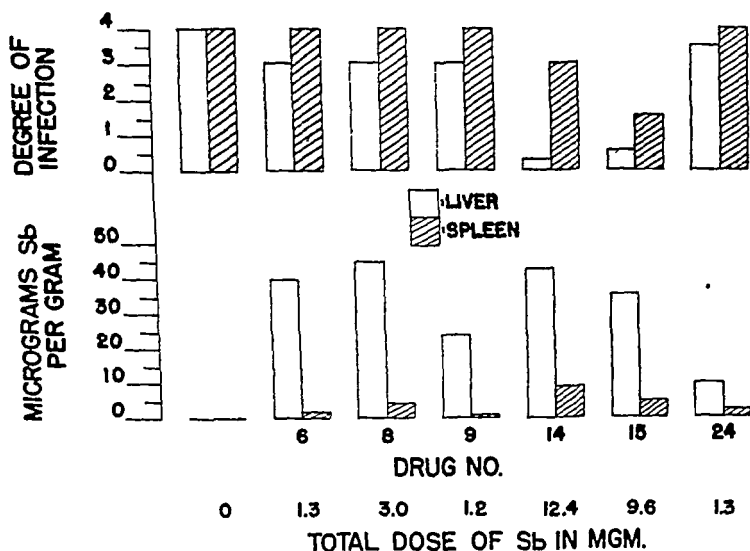


FIG. 2. COMPARISON OF THE ANTIMONY CONTENT OF LIVER AND SPLEEN AND CHEMOTHERAPEUTIC EFFECT OF VARIOUS TERVALENT ANTIMONIALS

since it was not feasible to determine the antimony content of the bone marrow or lymph nodes in the hamster, the correlation between antimony content and therapeutic effect was limited to the liver and spleen.

From the results of the experiments it is clear that there is a partial correlation between the concentration of antimony in the spleen and the therapeutic result. Thus in those instances where there was a negligible concentration of splenic Sb, there was no apparent reduction of parasites in the spleen. On the assumption that it is the antimony which destroys the parasite and not either the organic molecule or the host's own mechanisms of resistance stimulated by the drug as suggested by Napier and Mullick (12), the correlation of low antimony concentration and low therapeutic activity is not surprising. The results then would justify the conclusion that a certain concentration of antimony is necessary in a

tissue before a chemotherapeutic effect may be anticipated. However, as can be seen from table 2 and figure 3, the observations with Stibacetin (drug No. 20) demonstrate that a high Sb concentration is not sufficient to obtain a good thera-

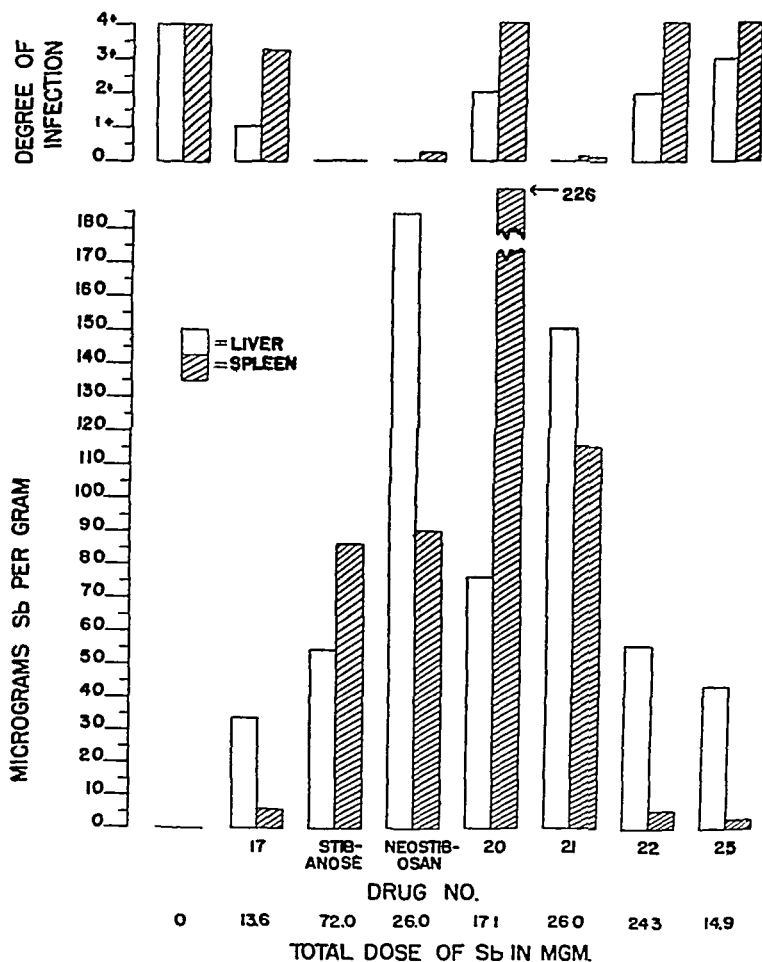


FIG. 3. COMPARISON OF THE ANTIMONY CONTENT OF LIVER AND SPLEEN AND CHEMOTHERAPEUTIC EFFECT OF VARIOUS QUINQUEVALENT ANTIMONIALS

peutic response. Although the concentration of Sb in the spleen is higher following the administration of this drug than following the injection of any other compound, no appreciable decrease in the parasitization of this organ was dis-

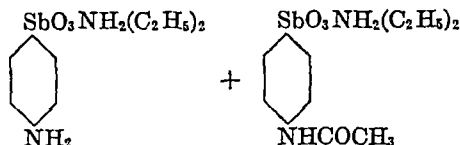
cernible. Also it is to be noted that in many instances a high antimony concentration in the liver was not sufficient to produce a chemotherapeutic effect. This may reasonably be explained by the hypothesis that if the spleen is not cleared of the leishmania the liver is constantly reinfected due to the intimacy of the splenic and portal circulations so that irrespective of the antimony concentration in the liver, the parasites cannot be eradicated from this organ.

In addition to the partial correlation between the therapeutic activity of the drugs and the concentration of antimony in tissues, it is instructive to correlate the presumed chemical structure of the most effective drugs, quinquevalent antimonials, with their therapeutic activity. This has limited value owing to two facts: (1) none of the drugs used was pure and one, Neostibosan, is a mixture to

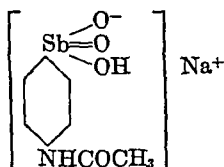
Group A

Drug

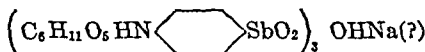
Neostibosan



20 Stibacetin



21 Neostam



a particular part of which neither tissue concentration nor toxicity could be attributed with certainty; (2) the limited data were all gathered in hamsters and would not necessarily be similar in other animals including man. If these qualifying statements are kept in mind, one can arrange the drugs into two groups (A) substituted phenylstibonic acids such as Neostibosan and, (B) other quinquevalent antimonials in which the metal is linked twice through oxygen or sulfur to carbon.

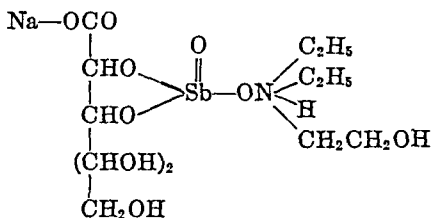
Judged by the chemotherapeutic test employed in the present investigation, Stibacetin (drug No. 20) although it was once used for the treatment of human visceral leishmaniasis, is the only inactive member of Group A. In the absence of synergism, one may conclude that its presence in the mixture marketed as

Neostibosan adds nothing to the activity of the mixture. By difference, then, the remaining position of Neostibosan, viz., the p-aminophenylstibonic acid (present as the diethylammonium salt) is active. Hydrolysis of the glucosidic

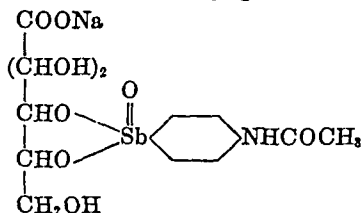
Group B

Drug

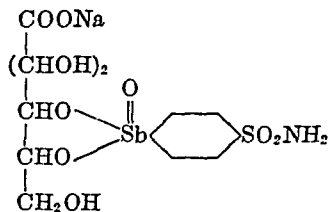
Stibanose



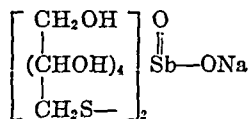
22 Sodium 4-acetylaminophenylstibonyl gluconate



25 Sodium p-sulfonamidophenylstibonyl gluconate



17 Sodium antimony thiosorbitol



linkage of Neostam, which presumably occurs readily *in vivo*, would also liberate p-aminophenylstibonic acid. Consequently, it would appear that substances of this type owe their therapeutic activity to the presence of free or potential

p-aminophenylstibonic acid, and this activity is lost if the p-amino group is acetylated.

Among the drugs of Group B, only Stibanose is chemotherapeutically active. Chemically, Stibanose is like p-aminophenylstibonic acid in that it is a stibonic acid. It differs, however, in that it is not linked directly to carbon, but is linked twice through oxygen. Probably the antimony in No. 17 is bound more firmly to the organic moiety because of its linkages through sulfur; therefore, it may owe its inactivity to the slow rate of transfer of antimony from the drug to the parasitic protoplasm. Apparently the chemotherapeutic activity of the Stibanose type of molecule is conditioned further by the presence of an electrovalence for compounds 22 and 25, which differ from Stibanose mainly in that the electrovalence is replaced by a covalence which may account for their lack of activity.

If one were to assume that the mechanism of action of drugs of the Stibanose type requires a transfer of antimony from the gluconic acid moiety (and this would be equivalent to the liberation of the antimononic acid) then it would be reasonable to assume that compound No. 22 would generate Stibaceticin, which we have already shown to be inactive. However, drug No. 22 does not lead to a high splenic concentration of antimony. Similarly drug No. 25 might liberate p-sulfonamido phenylstibonic acid, which, one might infer from the discussion of Group A would probably be inactive.

SUMMARY

The distribution of the antimony of 8 tervalent and 7 quinquevalent antimonials in liver and spleen was studied in hamsters infected with the Khartoum strain of *L. donovani* for the purpose of correlating tissue distribution with chemotherapeutic effect. Only 3 compounds, all quinquevalent, were effective and in all 3 instances there was a high splenic concentration of antimony (about 90 micrograms per gram). In all the remaining drugs (except one) whether tervalent or quinquevalent, the splenic concentration of Sb was 10 micrograms per gram or less. The behavior of the quinquevalent drug, Stibaceticin, was exceptional in that its antimony was deposited in the spleen in very high concentration (226 micrograms per gram) and yet it showed no chemotherapeutic activity. It is concluded that by the chemotherapeutic test used, a high splenic concentration of antimony is a necessary but not a sufficient condition for the cure of experimental leishmaniasis.

The correlation between the structure of quinquevalent antimonials and chemotherapeutic activity is discussed.

The technical assistance of Miss N. A. Tupikova is gratefully acknowledged.

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SOME TOXICOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF CITRININ*

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Since the introduction of the sulfonamides as antibacterial compounds, increasing interest has been directed to the discovery of new chemotherapeutic agents. The discovery of penicillin by Fleming has opened a new field of investigation on antibacterial substances produced by the metabolism of molds. These antibiotics have been shown to possess marked bacteriostatic and bactericidal properties against Gram-positive and in some instances against Gram-negative organisms (Dubos, Waksman and Woodruff) (1).

Citrinin is an antibiotic produced by *Penicillium citrinum*. Its preparation from pure culture media, and physical and chemical properties have been described by Hetherington and Raistrick (2). The bacteriostatic and bactericidal properties have been reported by Raistrick and Smith (3), by Oxford (4), and by Tauber, Laufer and Goll (5). Recently Timonin and Rouatt (6) have reported the isolation of citrinin from the culture media of *Aspergillus Sp.* of the Candidus group. The product isolated in crystalline form has been found to have the same properties as that isolated by Hetherington and Raistrick.

Citrinin has been reported to exhibit bacteriostatic properties against staphylococci in dilutions of 1:15,000 to 1:50,000. Against *Staphylococcus aureus* and *Staphylococcus albus* it has been shown to be bactericidal in a dilution of 1:8,000. Citrinin has about one six hundredth the bacteriostatic power of penicillin. However, it is much more stable, easier to isolate in pure form, and the yield per unit of culture medium is decidedly greater.

To our knowledge no studies have been reported on the pharmacology of citrinin, other than limited toxicological data by Timonin and Rouatt (6), Robinson (7), and more recently by us (8). In the present communication studies will be presented on the isolation and purification of citrinin from a natural culture medium, some chemical properties, additional toxicological data, and certain pharmacological properties.

ISOLATION AND PURIFICATION. Crude citrinin was made available to us through the cooperation of the microbiological section of the Biochemical Division of the Western Regional Research Laboratory. *Penicillium citrinum* Thom was grown on asparagus-butt juice media, as described by Humfeld and Feustel (9). Crude citrinin was obtained by adding concentrated hydrochloric acid to pH 3 to the filtered metabolic solution. A dirty yellow precipitate forms rapidly at this pH which can be readily collected by the usual procedures. This precipitate was contaminated with other substance(s) which for the most part can be separated by extraction.

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The crude precipitate of citrinin was dried, triturated and exhaustively extracted with hot ethyl acetate, in which it is readily soluble. The ethyl acetate extract was concentrated in vacuum to about one-half its original volume. On cooling, orange-yellow crystals separated out and were collected on a Buchner funnel. A second crop of crystals may be collected by further concentration of the mother-liquor, but this is inadvisable since more impurities tend to come down in the second crop of crystals than in the first. The ethyl acetate insoluble residue, which has a clay color, was saved for future study. The citrinin so obtained is still contaminated with a reddish pigment, presumably oxidation products, which for the most part can be removed by repeated recrystallizations from ethyl alcohol. By this procedure we have been able to recover about 75 per cent of the crude material in the form of pure citrinin.

The dry citrinin crystals as obtained by ethyl acetate extraction were dissolved in a minimum amount of hot aldehyde-free ethyl alcohol. As soon as the solution was complete, the vessel containing citrinin was placed in a vacuum desiccator for crystallization. The crystalline material was collected on a Buchner funnel, washed with water and dried. The melting point was not altered by further recrystallization from hot ethyl alcohol, dioxane, acetone, ethyl acetate, ethyl ether, or by dissolving in sodium hydroxide and precipitating with hydrochloric acid. This sample of citrinin together with one prepared by sublimation

TABLE I
Solubility of Citrinin at 25°C. in Various Solvents

SOLVENT	QUANTITY DISSOLVED GRAM/100 ML.
1. Petroleum ether (B.P. 35°-60°)	0.056
2. Ethyl ether	0.578
3. Ethyl alcohol (95%)	0.703
4. Ethyl acetate (B.P. 77°)	2.11
5. Benzene (B.P. 80°)	2.858
6. Acetone	4.94
7. Chloroform.	7.74
8. Water	Insol.

by Dr. F. T. Jones of the Physiochemical and Analytical Division of the Western Regional Research Laboratory was used in most of the tests outlined below.

GENERAL PROPERTIES OF CITRININ. The solubility of citrinin was determined at 25°C. in various solvents listed in table I. In determining the solubility, citrinin in excess was added to about 25 ml. of the respective solvent in a glass-stoppered cylinder and shaken for one hour. The solution was filtered and a measured aliquot was evaporated to dryness and constant weight in a wide-mouth tared weighing bottle.

The solubility of citrinin in solvents of higher boiling points was not determined, since it appeared to us that the higher-boiling point solvents tended to yield crystals of citrinin with a reddish color on removal of the solvent. In hot solvents the solubility of citrinin was greatly increased. In hot ethyl alcohol the solubility was about 5 grams per 100 ml. No direct estimation was made, but we have dissolved 20 grams of citrinin in 100 ml. of hot ethyl acetate without any difficulty, indicating that solubility had increased at least ten-fold.

Citrinin crystallizes from organic solvents in lemon-yellow glistening prismatic crystals. When exposed to the rays of an ultra-violet lamp citrinin fluoresces greenish yellow. Citrinin forms a sodium salt when added to a solution of sodium hydroxide or acetate. However, in such alkaline solutions citrinin readily undergoes a change, presumably an irreversible oxidation, and the more alkaline the solution the more rapid is the change. A

freshly prepared 5-per cent solution of citrinin at pH 7.4 has a light lemon-yellow color, about the same as that of a 2-per cent ferric chloride solution. After standing several days at room temperature or at 5° C. a reddish color gradually develops, the change being more rapid at room temperature than at the lower temperature. At pH 6.9 solutions of citrinin do not deteriorate as rapidly as those on the alkaline side, but in time they too will develop a reddish color.

In a previous publication (8) we have presented data on melting points and on carbon and hydrogen contents of two samples of citrinin, one purified by sublimation and the other by extraction. These data are in substantial agreement with those reported by Hetherington and Raistrick (2) and with the theoretical carbon and hydrogen contents.

Microbiological tests against *Staphylococcus aureus* were made by Dr. Humfeld of the Biochemical Division on citrinin purified by extraction and by sublimation, on the ethyl acetate-insoluble residue, and on the nonvolatile residue. No difference in activity was found between the two samples of citrinin. The insoluble and nonvolatile residues showed no antibacterial activity. These observations are in harmony with the toxicity data on laboratory animals reported below.

ACUTE TOXICITY. Studies have been made on the acute toxicity of citrinin after subcutaneous, intraperitoneal or intravenous injection in rats, mice, guinea pigs, and rabbits.

Two samples of citrinin were used in these experiments, one purified by sublimation and the other by solvent extraction as described above. Solutions of citrinin were prepared immediately before use by dissolving weighed amounts of each sample in N-sodium hydroxide and adjusting the pH of the solutions to approximately 7 with the aid of a glass electrode.

The acute toxicity of citrinin was determined subcutaneously in 53 rats, intraperitoneally in 41 rats, subcutaneously in 17 guinea pigs, intravenously in 31 rabbits, and subcutaneously or intraperitoneally in 131 mice. The animals were observed frequently during the first 6 hours after injection, and thereafter twice daily for 14 days. Autopsies were performed soon after death whenever possible, and sections of various organs were taken out for histological examination.

Since most of the toxicity data have been published (8) only the approximate LD₅₀ for the 14-day period need be given here. The toxicities of the two samples of citrinin were essentially the same, and the data were combined. The LD₅₀ for rats, by both routes of administration, was 67 mgm.; for guinea pigs 37 mgm.; for rabbits 19 mgm.; and for mice 35 mgm./kgm.

Various symptoms developed following suitable doses of citrinin. These may be described in general, since the differences in symptoms were dependent upon the size of the dose, and in some cases on the route of administration.

In rats, one of the most striking and consistent observations made was the intense hyperemia of the ears and feet, which developed within 30 minutes after administration of citrinin. The onset of hyperemia did not appear dependent upon the dose or the route of administration, but the intensity was more marked with the higher doses, and usually lasted for about two hours.

In rats receiving 50 mgm. or more subcutaneously, other less consistent symptoms, such as, respiratory stimulation, dyspnea, or prostration, were present.

After intraperitoneal administration in doses of 50 mgm. or more, besides the symptoms enumerated above, watery discharge from the nares was not an infrequent observation in most of the animals. By the following day all of the survivors, with few exceptions, appeared normal. However, for about a week after the administration of citrinin all of the rats, with the exception of those receiving 25 mgm., consumed less food and water, their intake being about one-half of normal.

In rabbits receiving citrinin intravenously in doses of 10 mgm./kgm. or more, the two outstanding symptoms were miosis and hyperemia of the ears. While these symptoms were present in all rabbits, the effects were more pronounced in those receiving the higher doses. Rabbits receiving 15 or 20 mgm. exhibited salivation, lacrimation and frothy discharge from the nares. Other less striking and less consistent symptoms were defecation, micturition, and respiratory stimulation. The administration of atropine locally or intravenously to rabbits before or after the injection of citrinin was without effect on the miosis.

On guinea pigs and mice the effects on respiration were the same as those observed in rats and rabbits.

Two rabbits received intravenously 25 and 50 mgm./kgm., respectively, of the ethyl acetate insoluble residue, and two other rabbits were given intravenously 15 and 30 mgm./kgm., respectively, of the nonvolatile residue. In no instance were any toxic symptoms produced.

SUBACUTE TOXICITY. The effect of repeated intravenous injections of citrinin was studied in 6 rabbits. Daily injections, except for Sunday, were continued for 8 weeks. The rabbits were weighed once each week and the daily dose for each animal was computed from these weights. Daily observations were made on food and water consumption as well as on the general behavior of the animals. Observations were made of rectal temperatures, red and white blood cell counts and hemoglobin values, and tests for sugar and albumin in the urine were made at frequent intervals.

Three of the 6 rabbits received 5 mgm./kgm. of citrinin daily for 26 days. The dosage was raised to 15 mgm./kgm. for the next 13 days and finally to 20 mgm./kgm. for the last 13 days. The other 3 rabbits were given 10 mgm./kgm. daily for 26 days, and 20 mgm./kgm. daily for the next 26 days. There were no fatalities in these experiments; and 24 hours after administration of the last dose all rabbits were killed by a blow on the head. Gross examinations of the tissues at autopsy showed no abnormalities.

Miosis and hyperemia of the ears were the most striking and consistent effects noted in these rabbits. Rabbits receiving 10, 15, and 20 mgm./kgm. of citrinin frequently exhibited salivation, lacrimation, and frothy discharge from the nares. All the above symptoms were more marked on the higher doses, appeared shortly after the injections and lasted for about one-half hour. In some rabbits, irrespective of the dose administered, there was some evidence of irregular respiratory effects; in some instances respiration was stimulated, while in others it was depressed. Usually the symptoms appeared more exaggerated after the first injection following a 24-hour rest period (Sunday), and became less pronounced after each subsequent injection during the week, but never to the point of

extinction. This diminution in severity of symptoms suggests tachyphylaxis.

No significant changes were observed in rectal temperatures over a two-hour period following the injections of citrinin. Urine examinations for sugar and albumin were consistently negative for all six rabbits.

The effect on the blood picture is shown in table 2, which lists the red and white cell counts and hemoglobin values for the six rabbits before administration of citrinin and on termination of the experiment. These data indicate that the blood changes are of no particular significance. While the hemoglobin values appear to indicate an effect, it should be noted that two of the three rabbits receiving the higher doses showed smaller changes than animals on lower doses.

PATHOLOGY. Histological studies were made on the tissues of one guinea pig dying after receiving 75 mgm./kgm. of citrinin, of two guinea pigs dying after receiving 50 mgm./kgm., of two rabbits dying after the intravenous injection of 20 and 50 mgm./kgm., respectively, and of six rabbits sacrificed after receiving sublethal doses of citrinin intravenously for eight weeks. All tissue sections studied were stained with hematoxylin-eosin.

TABLE 2

The effect of daily administration of citrinin on erythrocyte, leucocyte and hemoglobin values in rabbits

RABBIT NO.	TOTAL DOSE OF CITRININ*	ERYTHROCYTES		LEUCOCYTES		HEMOGLOBIN	
		Before	After	Before	After	Before	After
	gms.	millions				gms./100 ml.	gms./100 ml.
1	1.37	7.24	6.29	9650	12150	15.0	11.9
2	1.45	6.46	5.04	9000	9450	11.2	10.9
3	1.63	5.00	5.02	9200	6250	13.1	9.9
4	1.79	5.88	5.65	7750	10700	11.3	10.8
5	1.95	5.72	6.20	7800	7050	12.3	12.0
6	2.29	7.28	6.54	4750	5000	14.1	11.8

* First three animals received small initial dose; remainder received large initial dose.

In the animals receiving 75 or 50 mgm. of citrinin, marked changes were found in the kidneys, consisting principally of swelling, necrosis, and desquamation of the tubular epithelium. These changes occurred largely in the proximal convoluted tubules. There were many tubular protein casts, and a few clumps of desquamated epithelial cells stained with hematoxylin, suggesting calcification. Some of the non-necrotic tubular epithelial cells had clear vacuolated cytoplasm, suggesting that they may have stored some substance which was dissolved out during preparation of the tissue sections. The glomeruli appeared structurally normal. The hearts of all but one of these animals showed a few small foci of acute myocarditis. The lung from one guinea pig showed unusually marked collapse, and the stomach showed marked necrosis of portions of the mucosa, but these changes were not present in any other animal.

The rabbit receiving 20 mgm./kgm. of citrinin exhibited similar kidney lesions which were only slightly less severe. Several glomeruli showed sharply defined round masses of amorphous eosinophilic material mixed with many blood cells

at one side of the capillary tuft. These masses were as much as half of the diameter of a glomerulus. They were sometimes seen to be surrounded by a layer of cells resembling endothelium, and their structure suggested thrombi in greatly dilated capillaries. There was no acute myocardial lesion in this animal, although there were two small foci of proliferation of cells resembling fibroblasts.

All the rabbits receiving the smaller repeated doses of citrinin showed mild but definite kidney lesions of varying degree. These lesions were often localized to scattered small areas, between which the tubules appeared normal. The involved tubules were often dilated, lined in places by flattened cells, and elsewhere by smaller clear cells like those described above. Necrotic cells were not seen. Rabbit No. 1 (table 2), which received a total dose of 1.37 grams of citrinin, showed the fewest changes. Rabbits Nos. 4 and 6, which received total doses of 1.79 and 2.29 grams, respectively, showed the most abnormal kidneys. The other organs were not significantly different from those of the control animals.

In summary, then, all animals examined for pathological changes showed evidence of kidney injury, which occurred particularly in the proximal convoluted tubules. The severity of the kidney damage was roughly proportional to the dose of citrinin, but was not greater in the animals receiving prolonged administration of citrinin. The animals receiving the highest doses showed occasional small foci of myocarditis.

LOCAL EFFECTS. Since the sulfonamides, penicillin, tyrothricin and similar agents have found use clinically on mucous membranes and in the treatment of open wounds, there is reason to believe that if citrinin proves to have desirable therapeutic properties, it too may find a place in topical application. On the other hand, if it should have little therapeutic use, but prove to be a substance of economic importance for other reasons, it would be desirable to know what effects citrinin might have on those handling the compound. For these reasons the possible irritant properties have been studied.

Two and five per cent solutions of citrinin, pH 7.2, were instilled into the conjunctival sacs of albino rabbits, the contra-lateral eye serving as a control in each case. Observations were made at 15-minute intervals over a period of 4 hours, and again after 24 and 48 hours. Both concentrations of citrinin were irritant as judged by a slight degree of conjunctivitis and edema which lasted about two hours. On the following day these symptoms were absent. Dry citrinin, or the ethylacetate insoluble residue, dusted in the eye produced marked irritation of the conjunctiva characterized by edema and inflammation which was still present 24 hours after application. In no case was there any evidence of miosis, as was seen after parenteral administration of citrinin.

Three laboratory workers noted slight irritation of the nasal passages after unintentional inhalation of small amounts of citrinin dust. Segments of rabbit, rat, and guinea pig intestine, slit longitudinally and washed with 0.9-per cent sodium chloride to remove detritus, were suspended in 1-per cent solutions of citrinin at pH 7.2. They did not appear any different from control strips suspended in 0.9-per cent sodium chloride over a period of one hour.

Rats and guinea pigs receiving subcutaneous or intradermal administration of

a 2.5-per cent solution of citrinin in varying amounts showed no evidence of local irritation or necrosis. Intradermal injection of 0.1 ml. of a 2-per cent solution of citrinin at pH 7 into the left forearm of one of us (A. M. A.) caused no local irritation.

HEMOTOXIC EFFECTS. In the following experiments washed red blood cells from rabbits and rats were used, and the method of Sanford (10) was employed. The red cells were washed and diluted 1 to 4 with 0.9-per cent sodium chloride. The stock citrinin solution contained 10 mgm. per 100 ml. in 0.5-per cent sodium chloride. In one series of 6 tubes serial dilutions were made, ranging from 6 to 10 mgm. per 100 ml. of citrinin and from 0.3- to 0.5-per cent sodium chloride, respectively. In a second series of 6 tubes the sodium chloride concentration was kept constant at 0.5 per cent while the concentration of citrinin was varied as in the first series. Suitable controls without citrinin but containing the same salt concentrations and comparable numbers of red cells were run simultaneously.

In the first series hemolysis of rabbit and rat red cells occurred at the same salt concentrations as in the respective controls. Definite and complete hemolysis occurred in 0.34 and 0.38-per cent sodium chloride for rabbit and rat red cells, respectively. In the second series, where the salt concentration was kept constant at 0.5-per cent and the citrinin varied from 6 to 10 mgm. per 100 ml., no hemolysis occurred.

The test for agglutination was made by adding washed red cells to a citrinin solution containing 20 mgm. per 100 ml. in 0.5-per cent sodium chloride. Hanging-drop slides were prepared and examined microscopically over a period of four hours. No agglutination occurred.

In view of the other pharmacological properties of citrinin, to be discussed below, it is evident that any action on erythrocytes plays no significant rôle in the acute toxicity of this antibiotic. The absence of a hemotoxic or agglutinating action of citrinin differentiates it from tyrocidine, tyrothricin and gramicidin.

EFFECTS ON ISOLATED ORGANS. Studies were made of the action of citrinin on the rabbit and guinea pig intestines, on the uteri of rats and guinea pigs, on the gastrocnemius muscles of frogs, on frog and guinea pig hearts, and on perfused guinea pig lungs.

For the studies on intestine, isolated strips of rabbit and guinea pig ileum were used. Detritus within the lumen was washed out by allowing large quantities of warmed Ringer-Locke solution to flow through by gravity. Spontaneously active segments of rabbit intestine suspended in 50 ml. Ringer-Locke solution were not affected by citrinin in concentrations of 1:10,000. Concentrations of 1:5,000 produced depression of the amplitude of rhythmic contraction with a slight change in rate. In higher concentrations there was a depression of both contractility and tone. These higher concentrations of citrinin also prevented the action of acetylcholine, nicotine, and barium chloride. In the case of the guinea pig intestine, a citrinin concentration of 1:10,000 produced either no effect or a slight decrease in tonus. The action was not reversible, since upon removal of the citrinin and washing of the segments with fresh Locke's solution the contractions did not return to normal. From these results it appears that the action of citrinin is directly upon the smooth musculature. (See Fig. 1).

The uterine actions of citrinin were studied on rat and guinea pig uteri suspended in Locke's or Van Dyke and Hastings solution (11). On the rat uterus, citrinin in concentrations of 1:25,000 produced an increased tone and decreased motility and rate. The guinea pig uterus was not appreciably affected by citrinin in a concentration of 1:50,000, but a concentration of 1:25,000 caused a gradual increase in tone, which was antagonized by the addition of Trasentin, 1:10,000. This increase in tone was accompanied by a slight increase in motility and rate. However, continued exposure of the guinea pig uterus to a concentration of 1:25,000 citrinin gradually decreased tonus, motility and rate to a point where they were abolished. In a concentration of 1:10,000 there was a slight increase

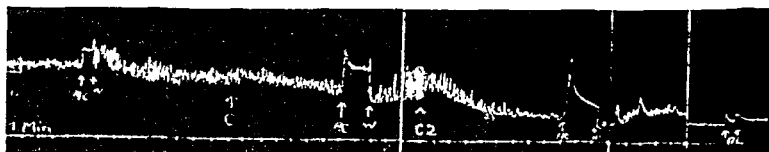


FIG. 1. EFFECT OF CITRININ ON THE RABBIT INTESTINE

At C and C2, citrinin was added to the bath to make the final concentration 1:10,000 and 1:5,000 respectively. At AC, acetylcholine was added to final concentrations of $1:2.5 \times 10^7$. At W the preparation was washed with fresh Locke's solution. Time interval, 1 minute. Breaks in record, 20, 3 and 2 minutes respectively.

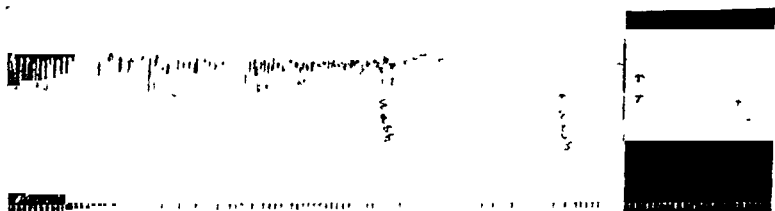


FIG. 2. EFFECT OF CITRININ ON THE ISOLATED VIRGIN GUINEA PIG UTERUS

At C and C2, citrinin was added to make the final bath concentration 1:25,000 and 1:5,000 respectively. W, indicates washing with Van Dyke and Hastings solutions; T, Trasentin was added to make 1:10,000 solution. Time interval, 1 minute. Break in record, 35 minutes.

in tone and rate which was abolished by atropine in a concentration of 1:2,500. A definite increase in rate and tone was produced by a 1:5,000 concentration of citrinin. These observations on the guinea pig uterus are quite different from those reported by Van Dyke (12) for the sodium salt of penicillin where only a "trivial single contraction" was produced by a concentration of approximately 1:5,000. (See Fig. 2).

The frog gastrocnemius was used to study the action of citrinin upon skeletal muscle. Muscle preparations were set up in pairs, suspended in Locke's solution, and arranged for independent or simultaneous faradic stimulation. The threshold of stimulation was first determined for each muscle. One muscle was kept as a control and to the bath of the other citrinin was added to make a final

concentration of 1:10,000. Both muscles were given simultaneous faradic stimulation of sufficient duration to produce an effect which was recorded on a slow moving kymograph paper. Stimulation was repeated at 5 minute intervals. Within 10 minutes the muscle in the citrinin solution no longer responded to the same stimulus to which the control muscle responded. Moreover, there was evidence of shortening of the citrinin-treated muscle. After about 90 minutes the treated muscle no longer responded to stimuli of any intensity within the range of the inductorium (Harvard type), while the control muscle responded to the threshold stimulus. The treated muscle was now definitely shortened. Repeated washing of both the control and treated muscle with Locke's solution did not relieve the contracture in the treated muscle.

The cardiac effects of citrinin were studied on isolated and intact frog hearts, and on the isolated guinea pig heart. Isolated frog hearts were prepared for perfusion according to the method of Straub, a cannula with a side arm being used instead of a Fuehner heart cannula. In all cases the hearts were rendered hypodynamic by frequent washing with Ringer's solution. After clamping the cannula with heart attached to a ring stand, the apex of the ventricle was connected to a suitable writing lever for kymographic recording. Perfusion was continued by means of a siphon connected to a reservoir of Ringer's solution. Means were provided for aerating the perfusion solution in the cannula. The perfusion pressure was maintained at 4 cm. of water and the rate of flow was adjusted to about 3 ml. per minute. The doses of citrinin were added to the perfusion fluid as it entered the aortic cannula.

Doses of citrinin smaller than 1 mgm. were without effect. With 1 mgm. there was a moderate increase in amplitude of the heart beat with no change in rate. The effect of citrinin on the isolated heart was much like that produced by a suitable dose of epinephrine, only more sustained.

The only noteworthy change produced in the frog heart in situ by 1 mgm. doses was a slight increase in the amplitude of contractions. With 2 mgm. the increase in amplitude was greater, but the heart ultimately stopped midway between diastole and systole.

The isolated guinea pig heart was perfused through the coronary vessels with Ringer-Locke's solution at 37°C. through a cannula tied into the aorta. The apparatus described for perfusing lungs, with slight modification, was used. The resistance of fluid passing through the pulmonary artery and the ventricular contractions were recorded simultaneously. (See Fig. 3).

The effects of citrinin on the isolated mammalian heart were essentially the same as those seen on the isolated and intact frog heart. Doses of less than 1 mgm. injected into the cannula directly above the aorta were without effect on the coronaries, or on ventricular amplitude and rate. Doses of 2 mgm. and over produced marked ventricular contraction which was invariably followed by coronary dilatation. Whether or not the dilatation was preceded by constriction is not clear.

The rate of flow through the coronaries was a determining factor. When 2 mgm. of citrinin was injected and the perfusion rate was about 5 ml. per minute the effects were more pronounced, while in hearts where the rate of flow was

about 60 ml. per minute the effects were demonstrable only after the injection of larger doses. The data suggest that only large doses of 0.2 mgm. or more of citrinin per ml. of perfusion solution would have any effect on the heart. Under conditions of therapeutic use it is unlikely that this concentration of citrinin would ever reach the intact heart.

The tachyphylactic nature of the response to citrinin was exhibited in the perfused hearts. Repeated additions of citrinin to the perfusing fluid caused decreasing responses to a point where no further effect was demonstrable. However, such hearts still responded to epinephrine.

It appeared probable that citrinin would cause bronchial constriction in view of the demonstrated action on other structures. For testing such action isolated guinea pig lungs, with or without the heart attached, were used. The perfusion technique devised by Sollmann and von Oettingen (13) and by Thornton (14) for excised lungs was used with

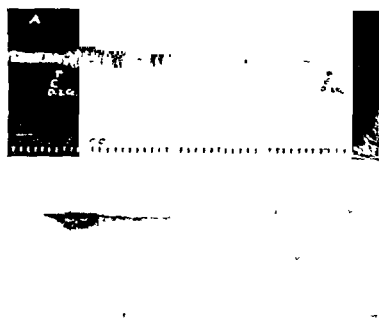


FIG. 3. EFFECT OF CITRININ ON THE HEART

Drugs introduced directly into the perfusion cannula:

A—Frog heart in situ, perfused with Ringer's solution; at C, citrinin 0.2 ml. of 1% solution; at E, epinephrine 0.2 ml. of 1:200,000 solution. Time interval, 5 seconds.

B—On the isolated guinea pig heart, perfused with Ringer-Locke solution. At E, epinephrine 0.1 ml. of 1:200,000; at C, citrinin 2, 1, 2 and 5 mgm. Figures above ventricular contractions represent coronary constriction or dilatation in cm. of water. Time interval, 1 minute. Flow, 5 ml./minute.

certain modifications. Briefly, the procedure was as follows: The heart and lungs of a freshly killed guinea pig were removed in toto and hung on a suitable perfusion cannula inserted into the trachea. The cannula with lungs attached was joined to one of two openings at the lower end of a water manometer tube. The second opening was joined to a reservoir of perfusion fluid, usually placed at a height to give a head of pressure of 45 cm. of water. The lungs were distended with the perfusion fluid and the pleura scratched lightly to permit escape of fluid from the alveoli. By means of a screw clamp between the reservoir and the tracheal cannula the rate of perfusion was adjusted to about 30 ml. per minute, giving an average perfusion pressure of approximately 20 ml. of water. At a constant head of pressure the rate of flow of the perfusion fluid through the lungs is influenced by the bronchioles and alveoli; with an increase in the rate of flow (dilatation) the pressure in the water manometer falls and, conversely, with decreased flow (constriction) the pressure rises. These changes were recorded graphically on a slow moving kymograph by means of a sensitive volume recorder attached to the open end of the manometer tube. The perfusing fluid, Van Dyke and Hastings (11), was warmed to 35°C. before passing into the lungs.

Histamine 1:10,000, acetylcholine 1:5,000, or epinephrine 1:1,000 was used to test the functional state of the bronchial musculature before and after the addition of citrinin. A 2-per cent solution of citrinin was made up in normal sodium hydroxide and the pH adjusted to approximately that of the perfusion fluid. All test agents were added to the perfusion fluid by injection through the rubber tubing connection proximal to the tracheal cannula.

In 25 guinea pig lung preparations citrinin was added to the perfusion fluid under a variety of conditions. In doses of 5 mgm. citrinin invariably caused constriction, which was relaxed by epinephrine. Here again tachyphylaxis was suggested by the fact that repeated additions of citrinin resulted in decreasing responses until a point was reached where the bronchial musculature no longer responded. However, the responses to histamine or acetylcholine re-

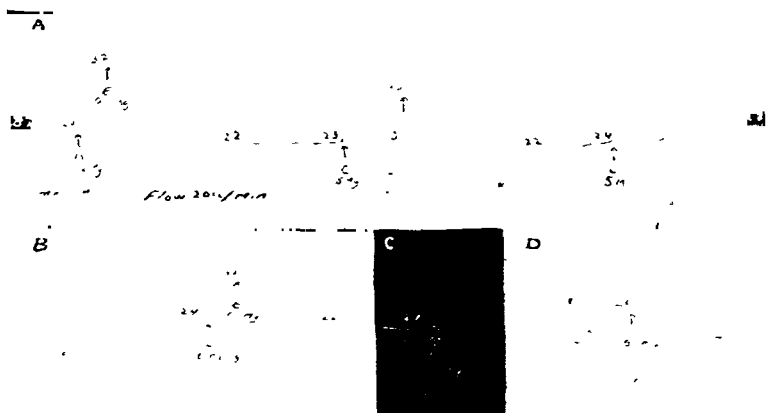


FIG. 4. EFFECT OF CITRININ ON THE ISOLATED GUINEA PIG LUNG

Records A, B, C, and D are continuous tracings. Break between A and B, 45 minutes, between B and C, 32 minutes and between C and D, 10 minutes. Drugs injected into tracheal cannula. At H, 0.02 mgm. histamine; at C, 5 mgm. citrinin, in record A and B, and 10 mgm. in record C. At E, 0.1 mgm. epinephrine. Numbers above the tracing indicate bronchial resistance (B.R.) in cm. of water, the larger the number, the greater the resistance to flow. Time interval, 1 minute; flow 20 ml./min.

mained normal. Repeated, small, ineffective doses of 1 or 2 mgm. of citrinin caused desensitization to doses of citrinin known to produce bronchial constriction. In suitable doses the simultaneous administration of citrinin with histamine, acetylcholine, or epinephrine did not inhibit or enhance the effects of the latter drugs. (See Fig. 4).

CIRCULATORY AND RESPIRATORY EFFECTS. The action of citrinin upon blood pressure and respiration was studied in 13 normal mongrel dogs under barbital or ether anesthesia. Blood pressure was recorded from a carotid artery with a mercury manometer. Respiratory movements were recorded by means of a tambour connected to the tracheal cannula. Both vagi were exposed either for faradic stimulation or for sectioning. All injections were made into a femoral vein connected to a burette through a cannula. All injections were followed by 10 ml. of 0.9-per cent sodium chloride. A freshly prepared 5-per cent solution of citrinin pH 7.1 was used in these studies. All doses given refer to mgm. per kgm. of

bodyweight, unless otherwise indicated. The functional state of the dogs was determined with suitable doses of epinephrine, histamine, pilocarpine, or acetylcholine.

In all dogs under barbital or ether anesthesia the circulatory and respiratory responses to citrinin were essentially the same. In doses of 5 mgm., citrinin produced an evanescent fall and recovery in blood pressure with no significant change in heart rate. The effect on respiration was inconstant; in some there was slight respiratory stimulation of short duration, while in others there was no apparent effect. Usually the stimulation of respiration was associated with the fall in blood pressure, and might therefore be attributed to a reflex of the carotid sinus. After denervation of the carotid sinuses and sectioning of the vagi no respiratory stimulation was apparent. On repeated administration of citrinin tachyphylaxis was seen in all dogs irrespective of the anesthesia. Each succeeding dose producing a lesser fall or rise (atropinized or vagotomized) than the previous dose, until after about the fourth injection the effect on blood pressure was only slight.

In dogs receiving larger initial doses, 10 or 20 mgm., the fall in blood pressure was no greater than that observed after 5 mgm. On these doses the fall and recovery of blood pressure was as rapid as with 5 mgm., but the blood pressure usually assumed a new level, 10 or 20 mm. below the control level. The depressor effect of citrinin was either annulled or reversed by atropinization or sectioning of both vagi. In this respect citrinin resembles acetylcholine, the muscarinic effects of which are abolished by atropin.

The drop in blood pressure caused by citrinin was very rapid, a fall of as much as 70 mm. of mercury being noted in less than 10 seconds. The recovery phase lasted longer but was usually complete within 2 minutes. This was particularly true after doses of 5 mgm. With the larger doses the drop in blood pressure was equally rapid, the recovery being somewhat more prolonged and usually 10 to 20 mm. below the control level, but returned to normal after epinephrine. The heart rate, on all doses, was not significantly altered. The inconstant respiratory effects may be secondary to the vascular effect.

In the unatropinized dog the evanescent fall in blood pressure may have been due to a vasodilator action on the vessels. In the atropinized dog there was a reversal of this citrinin action which may have been due to a paralysis of the dilator elements or an antagonism of a muscarinic action. To determine the cause of the fall in arterial blood pressure, changes in organ volume were recorded by either a kidney or leg oncometer and sometimes both. The oncometric measurements showed that peripheral vasodilatation accompanied the drop in blood pressure.

In one dog the effect of citrinin on gastric and intestinal motility was also determined. On the injection of 5 mgm., gastric motility was increased and tonus decreased. After vagotomy 5 mgm. produced essentially the same effect. However, injection of 10 or 20 mgm. after vagotomy increased both the rate and tonus. (See Fig. 5).

The possible effect that citrinin might have upon the epinephrine response was determined by injecting 0.25 ml. of a 1:10,000 dilution of epinephrine simultaneously with 10 mgm./kgm. citrinin. The response which followed was typi-

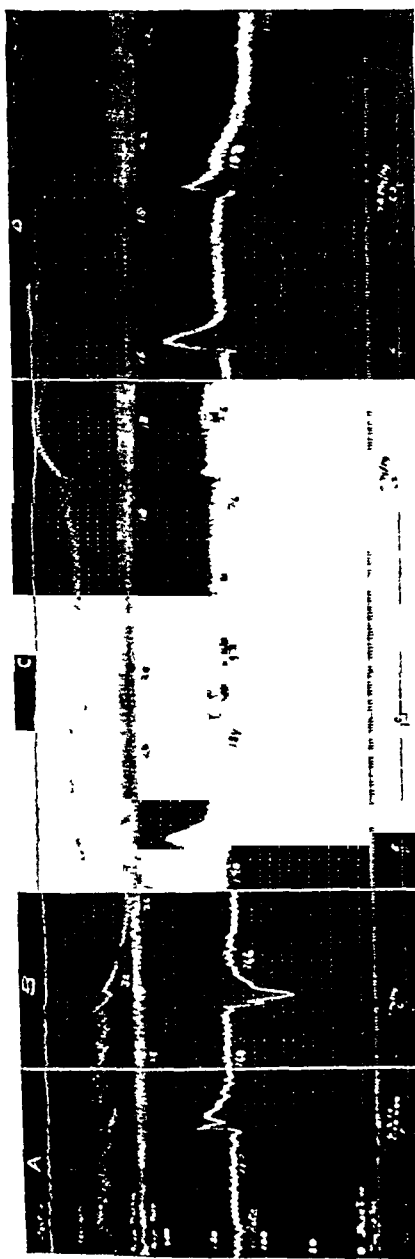


FIG 5 EFFECT OF CITRININ IN THE DOG

Dog 11.5 kgm anesthetized with barbital, 250 mgm /kgm I V. From above down, intestinal contraction, gastric motility, respiration recorded from tracheal cannula, respiratory rate per minute, time 10 second intervals, signal of injection. Injections made into femoral vein. At I, 0.2 cc 1 10,000 epinephrine, at C, C2 and C3, citrinin 5, 10 and 20 mgm /kgm. Breaks in record Between A and B, 15 minutes, between B and C, 25 minutes, and between C and D, 10 minutes. Between B and C, both vagi were cut and 15 and 25 minutes later citrinin was injected. Dog killed 20 minutes after last injections of citrinin.

cal of the epinephrine response as determined by a control test. No vasodepressor effect of citrinin was observed.

SUMMARY AND CONCLUSIONS

The effects of citrinin administered parenterally in suitable doses were typical of a parasympathetic stimulant. In rabbits intravenous injection of citrinin resulted in miosis, hyperemia, salivation, increased bronchial secretion, and lacrimation. These effects were not completely annulled by the doses of atropine used.

In dogs citrinin produced an evanescent fall in blood pressure, associated with a dilatation of the blood vessels of the skin, kidney, and extremities. Peristalsis of the stomach and intestine was increased. On the rabbit the ear vessels became widely dilated.

Studies of the action of citrinin on isolated organs revealed constriction of the bronchi, increased tone of cardiac muscle, decreased tone of the uterus, increased tone of the intestine, and increased tone of the frog gastrocnemius.

The various pharmacological actions of citrinin were characterized by tachyphylaxis.

It is interesting to note the similarity in action of citrinin, acetylcholine, and pilocarpine, despite the widely different structural formulae of the three compounds.

Acknowledgment: We are indebted to Dr. Alvin J. Cox, Jr., Department of Pathology, Stanford University School of Medicine, for the pathological studies reported herein and to Mr. Elton K. Doxtader of this division for technical assistance.

Note: Since the completion of this report, a paper by Wei-Chang Chu, on the same subject, has appeared in the *Journal of Laboratory and Clinical Medicine*, Vol. 31, p. 72, 1946.

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A COMPARISON OF DRUG-DIET THERAPY WITH SINGLE DAILY ORAL DOSAGE IN AVIAN MALARIA¹

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It is well established that in the use of the sulfonamides in the treatment of bacterial infections of animals or man, the administration of the drug in such a manner as to maintain, day and night, a more or less constant blood concentration is necessary to insure the best therapeutic effect. In the therapy of lophurae malaria in ducks, it has been shown that the same type of dosage schedule of sulfonamides is necessary for the most favorable therapeutic effect. In fact, it has been found that to obtain the same therapeutic effect over forty times as much sulfathiazole must be given daily in a single dose as is given by drug-diet therapy (1). It might be assumed that this principle of therapy is applicable to other drugs; however, the data reported here indicate that this is not always true. Each drug must be considered individually. When a single daily dosage is compared with drug-diet administration all possibilities may be encountered: single dose, less effective; single dose, equally effective; and single dose, more effective.

METHODS. All experiments have been done on lophurae malaria in the duck. The drug-diet method and the methods used in producing our infections and assessing the antimalarial activity of drugs have been previously described (1, 2). The single daily dosage was given in a volume of 10 cc. per kilogram by stomach tube with the drug in water solution or in suspension in 10-15 per cent acacia. Treatment was in all cases for six days. Birds were placed on the drug-diet or given the first daily dose 18 hours before infection. The doses per kilogram are calculated on the estimated average weight of the ducks during the seven days of the experiment.

RESULTS. A summary of the results is given in table 1. Here are given the minimal effective doses for drug-diet administration and for single daily dosage for nineteen drugs, and, in addition, the quinine equivalents of these drugs referred to the activity of quinine when given by the same method.

DISCUSSION. An examination of the data given in table 1 reveals that the relative therapeutic effectiveness of treatment by drug-diet as opposed to single daily dosage depends upon the particular drug in question. Thus, SN 4130, and SN 4271 resemble sulfathiazole in being much more effective when given by drug-diet; SN 11,437 is about three times as effective by drug-diet dosage. Quinacrine, SN 1452, SN 475, SN 6911, SN 8137, SN 10,286, SN 8557, and SN 13,710 are apparently equally effective with either dosage schedule. Quinine, cinchonine, pamaquine, SN 11,191, chloroquine, SN 7744 and paludrine are two

¹ This investigation was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University.

to three times as active, and SN 3115 is six times as active when given in single daily dosage.

TABLE 1

A comparison of the effectiveness of drug-diet administration with single daily dosage

SN*	NAME	MINIMAL EFFECTIVE DOSE		QUININE EQUIVALENT	
		Drug-diet	Single daily dose	Drug-diet	Single daily dose
		<i>mg./kg./day</i>			
359	Quinine†	27	8	1	1
1032	Cinchonine†	29	10	1	1
10286	7-chloro-2-phenyl- α -2-piperidyl-4-quinolinemethanol, dihydrochloride	3.7	3	8	3
13710	7-chloro-2-(p-chlorophenyl)- α -(dibutylaminomethyl)-4-quinoline methanol, monohydrochloride	1.7	1.6	16	5
971	Pamaquine†	0.4	0.15	64	53
1452	8-(3-aminopropylamino)-6-methoxyquinoline†	3	2	8	4
11191	8-(6-diethylaminoethylamino)-6-methoxyquinoline, diphosphate	0.4	0.2	64	40
3115	8-(3-diethylaminopropylamino)-6-methoxyquinoline, dihydriodide	1.7	0.3	16	27
6911	7-chloro-4-(4-diethylamino-1-methylbutylamino)-3-methyl quinoline, bisulfate	10	8	2	1
7618	Chloroquine, diphosphate	3.8	2	8	4
8137	1-(7-chloro-4-quinolylamino)-3-diethylamino-2-propanol, diphosphate	12	12	2	$\frac{1}{2}$
7744	4-tert-butyl- α -diethylamino-6-phenyl-o-cresol, hydrochloride	13	6	2	1
4130	Dimethyldithiocarbamic acid, 2,4-dinitrophenyl ester	60	3200	$\frac{1}{2}$	$\frac{1}{100}$
4271	Dimethyldithiocarbamic acid, methylene diester	26	1250	1	$\frac{1}{100}$
390	Quinacrine†	6	6	4	1
475	2,2',3,3'-tetramethyl-1,1'-diphenyl-[4,4'-bi-3-pyrazoline]-5,5'-dione	47	40	$\frac{1}{2}$	$\frac{1}{2}$
11437	N1-(-chloro-2-pyrimidyl)metanilamide	1.5	4.5	16	2
8557	2-[3-(decahydro-2-naphthyl)propyl]-3-hydroxy-1,4-naphthoquinone mixture of stereo isomers	8	6	4	1
12837	Paludrine, monohydrochloride	6	3	4	3

* SN is the Survey Number which will be used in a Monograph entitled "A Survey of Antimalarial Drugs, 1941-45" sponsored by the Committee of Medical Research of the Office of Scientific Research and Development.

† Used as a salt but calculated to base.

When the quinine equivalents² of a series of compounds are used to assess their potential antimalarial value for the human malaras, it is to be noted that

² The quinine equivalent is the ratio of the dose of quinine to that of the drug under test which produces the same effect on parasitemia.

different drugs may be selected depending on the dosage schedule used to determine the quinine equivalents. However, due to the lack of close correlation between activity in the avian and the human malaras (3) this is not of importance except in the case of compounds which are nearly inactive on single daily dosage but highly active when given by continuous administration, e.g., sulfonamides and dithiocarbamates.

SUMMARY

A comparison has been made of the relative antimalarial activity of nineteen drugs when given by single daily dosage as opposed to drug-diet administration. Depending on the particular drug used, all possibilities are encountered: single daily dosage, less effective; single daily dosage, more effective; single daily dosage, equally effective.

We wish to thank Charlotte Kennedy, Marjorie McBurney, Jean Hunt, Lucille Van Ghyl and Evelyn Epperson for technical assistance.

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THE EFFECT OF CHLOROFORM AND ETHER ON THE ACTIVITY OF CHOLINESTERASE

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Several attempts have been made to explain the parasympathetic phenomena associated with general anesthesia through an action on cholinesterase.

Adriani and Roverstine (1) reported that gaseous and volatile anesthetic substances do not inhibit the activity of cholinesterase, when added in vitro. They prepared saturated solutions of the anesthetic in human serum. They also investigated blood obtained from patients before and during general anesthesia. In these experiments the hydrolysis of acetylcholine was measured with the manometric apparatus of Van Slyke and Niell. On the other hand Bernheim and Bernheim (2) testing for the presence of acetylcholine pharmacologically by its action on a piece of guinea pig ileum or following the acetic acid production either by titration or manometrically, showed the esterase activity of the dog brain was inhibited by ether and chloroform. Torda (3) used a chemical method for testing the cholinesterase activity of ground cat muscle, and also found that chloroform and ether inhibit the activity.

Evidently there is no agreement in the evidence from different laboratories, and for that reason a number of experiments were carried out in an attempt to clarify the problem.

METHODS. For the determinations of the cholinesterase activity the Warburg manometric technic was employed. Ether and chloroform were the drugs used. Cholinesterase (specific) from the electric organs of the eel¹ and cat serum (non-specific) were employed as sources of the enzyme. The dilution of the enzymes were made in 0.03 M sodium bicarbonate. The acetylcholine bromide solution was made by dissolving 120 milligrams in 4.5 cc. of 0.03 M sodium bicarbonate. 0.5 cc. of this solution was placed in the side bulb. The ether and chloroform concentrations are shown in the tables. They were dissolved in the enzyme solutions, three cc. of which was placed in the bottom of each vessel, and allowed to stand from 60 to 100 minutes before the acetylcholine was tipped from the side bulb. The final dilution of the eel esterase was 1:972 and of the cat serum 1:10.2. Control vessels were run simultaneously in each experiment. The number of vessels used were three with drug and three controls. In the case of controls the same procedure was used except that the anesthetic drug was omitted from the enzyme solutions. Two thermobarometers were also included in each run. One contained the materials used in the vessels with the drug, and the other the materials used in the control vessels—the only difference being that acetylcholine was omitted in both thermobarometers. The flasks were equilibrated with a mixture of 5 volumes of carbon dioxide and 95 volumes of nitrogen in some experiments, but in most of them air was employed. The bath was maintained at 25° C. because of the volatility of the anesthetic drugs. A higher concentration of the enzymes than that usually used was made necessary by the low temperature, and also because the cat serum has less cholinesterase activity than human serum. When the readings became uniform

¹ Supplied through the kindness of Dr. David Nachmansohn.

the vessels were tipped and readings made at ten minute intervals for a period of 60 to 90 minutes. The straight line portion of the hydrolysis curve over a period of 30 minutes was used for the calculation of the cholinesterase activity. The average number of cubic millimeters of carbon dioxide liberated in the 30 minute period was determined for both sets of vessels. The esterase activity of the preparations containing ether or chloroform was expressed in percent of the activity of the control solutions.

RESULTS. Table 1 shows the results of eighteen experiments in which concentrations of ether and chloroform well in excess of those occurring in the blood during general anesthesia were employed. In these high concentrations both anesthetic drugs caused a definite inhibition of cholinesterase activity. For

TABLE 1

The effect of high concentrations of ether and chloroform on the activity of cholinesterase

EXP. NO.	DRUG	CONCENTRATION CC./100		GAS USED	ESTERASE	ACTIVITY IN % OF CONTROL
		Before tipping	After tipping			
8	Chlorof.	0.24	0.18	Air	Eel	91.90
9	Chlorof.	0.24	0.18	Air	Eel	67.91
13	Chlorof.	0.25	0.18	Air	Eel	81.04
14	Chlorof.	0.25	0.18	Air	Eel	62.07
15	Chlorof.	0.25	0.18	Air	Eel	67.29
16	Chlorof.	0.25	0.18	CO ₂ & N ₂	Eel	74.94
27	Chlorof.	0.26	0.22	Air	Cat serum	85.51
28	Chlorof.	0.26	0.22	Air	Cat serum	86.14
4	Ether	3.00	2.25	CO ₂ & N ₂	Eel	81.26
5	Ether	3.00	2.25	CO ₂ & N ₂	Eel	80.78
6	Ether	3.00	2.25	Air	Eel	76.88
7	Ether	3.00	2.25	Air	Eel	77.34
17	Ether	3.00	2.25	Air	Eel	80.10
18	Ether	3.00	2.25	CO ₂ & N ₂	Eel	85.36
29	Ether	3.00	2.57	Air	Cat serum	83.41
30	Ether	3.00	2.57	Air	Cat serum	80.11
34	Ether	3.00	2.57	Air	Cat serum	83.22
36	Ether	3.00	2.57	Air	Cat serum	81.34

ether the average activity was 81.0 per cent of the control value and for chloroform 77.1 per cent.

Eight additional experiments were carried out using a concentration of anesthetic approximating that found in the blood during the stage of deep anesthesia (4). There was no inhibition of cholinesterase activity (see table 2). Observations made in two cats in which the cholinesterase activity of the blood serum was determined before and during deep anesthesia with ether, also gave no evidence of a change in the cholinesterase activity. These results support the hypothesis that the effects of chloroform and ether are not mediated through an action on cholinesterase; however before a final conclusion can be drawn it would

be necessary to know the concentration of the anesthetic agent at the synapse and neuro-muscular junction and the sensitiveness of the cholinesterase associated with these structures to the agent.

Finally experiments were carried out to determine whether or not the inhibition resulting from high concentrations of ether is reversible. The enzyme-drug mixtures from two experiments (Nos. 34 and 36) were maintained at 37°C. for from three to four hours and the ether evaporated by passing air through the mixtures at intervals. The controls were similarly treated. Later these enzyme solutions were replaced in the vessels and the esterase activity determined.

TABLE 2

The effects of ether and chloroform in concentrations corresponding to those occurring in the blood during deep anesthesia

EXP. NO.	DRUG	CONCENTRATION CC./100		GAS USED	ESTERASE	ACTIVITY IN % OF CONTROL
		Before tipping	After tipping			
19	Chlorof.	0.03	0.026	Air	Eel	98.76
20	Chlorof.	0.03	0.026	Air	Eel	100.71
21	Chlorof.	0.03	0.026	CO ₂ & N ₂	Eel	99.36
26	Chlorof.	0.03	0.026	Air	Cat serum	98.33
22	Ether	0.20	0.15	Air	Eel	92.19
23	Ether	0.20	0.15	Air	Eel	106.21
24	Ether	0.20	0.17	Air	Eel	97.31
25	Ether	0.20	0.17	Air	Cat serum	99.53

TABLE 3

Reversibility of cholinesterase inhibition produced by ether

EXP. NO.	DRUG	CONCENTRATION CC./100		GAS USED	INCUBATED AT ROOM TEMP.	INCUBATED AT 37°	ACTIVITY IN % OF CONTROL	
		Before tipping	After tipping				Ether present	Ether removed
34-35	Ether	3.00	2.57	Air	minutes	minutes	83.22	89.17
36-37	Ether	3.00	2.57	Air	107	240	81.34	88.43

The results (see table 3) indicate some recovery of activity but it was not complete.

SUMMARY AND CONCLUSIONS

1) Ether and chloroform in concentration corresponding to those attained during deep general anesthesia do not inhibit the activity of cat serum cholinesterase *in vitro*.

2) In cats the cholinesterase activity of the serum during deep anesthesia was not depressed.

3) Ether and chloroform in concentrations higher than occur in blood during deep anesthesia inhibit cholinesterase *in vitro*.

4) The action of ether in the high concentration used is partially reversible.

5) These observations, while not conclusive, support the hypothesis that the parasympathetic effects observed during general anesthesia from ether and chloroform are not due to the inhibition of cholinesterase.

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AN APPLICATION OF THE DOSIMETRIC METHOD FOR BIOLOGICALLY ASSAYING INHALED SUBSTANCES

THE DETERMINATION OF THE RETAINED MEDIAN LETHAL DOSE, PERCENTAGE RETENTION, AND RESPIRATORY RESPONSE IN DOGS EXPOSED TO DIFFERENT CONCENTRATIONS OF PHOSGENE

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The biological analysis of volatile substances which enter the body through the respiratory tract has been complicated by the problems inherent in measuring the amount acquired by, and retained after, inhalation. Because of these technical difficulties, the effects of toxic vapors have been assayed in terms of the Haber equation (1)

$$Ct = K \quad (1)$$

in which C = the concentration of agent in micrograms/l. to which the animals are exposed in some type of gassing chamber, t = the time of exposure in min., and K = the concentration-time product or Ct in microgram-min./l. In accord with modern bio-statistical methods, it has become standard practice to express the toxicity of volatile agents in terms of the median lethal Ct or $L(Ct)50$; i.e., the Ct which would produce 50% mortality in an experimental population. Usually, to minimize the influence of possible temporal factors like detoxification, the time of exposure (t) is maintained constant, and the mortality is observed in groups of animals exposed to several concentrations (C).

When such $L(Ct)50$'s are compared as indices of toxicity, a wide range in susceptibility to many noxious vapors, for example, phosgene, is observed among the different species (2). Consequently, estimates of the toxicity for man, based on such studies of animal mortality, have failed to delimit sufficiently the probable range. In addition, when the concentration-time product is used as the measure of the dose of inhaled agent administered to members of a given species, marked individual differences in susceptibility have been reported; e.g., one mouse may survive exposure to phosgene at a Ct ten times as great as that which kills another (2).

Many investigators (3, 4, 5, 6, and 7) have felt that these reported species and individual variations in susceptibility are more apparent than real, resulting from the use of the concentration-time product, an inadequate experimental method for evaluating the toxicity of inhaled substances, which does not measure the actual dose retained after exposure. Actually, the amount of agent inhaled per unit of body weight during any exposure is given by the expression

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$$\frac{VCt}{w} = D \quad (2)$$

where, if V = respiratory volume per minute, c = concentration of agent in mgm./l., t = duration of exposure in minutes, and w = body weight in kgms., D = dose inhaled in mgm./kgm. (5, 6). However, not all the agent inhaled is retained, for some is expired with each exhalation. Consequently, to express quantitatively the dose of agent retained after inhalation, equation (2) must be modified to

$$\frac{VCt\alpha}{w} = D \quad (3)$$

where α = the percentage of agent retained, and the significance of the other symbols is the same as before (3, 4, 7).

A comparison of equation (3) with the Haber formula (1) makes it evident that the Ct product, as Haber undoubtedly recognized, constitutes a measure of the dose only if it is assumed that the percentage of inhaled agent retained (α) and respiratory intake per kilogram body weight $\left(\frac{V}{w}\right)$ are constant for all animals.

There is ample evidence that these assumptions are not valid. First, the respiratory minute volume per kgm. is not constant for all animals but, like the metabolic rate, increases somewhat as the total body weight decreases. For example, the resting 60 kgm. man breathes approximately 0.1 liter per kgm. per minute, whereas the 20 gm. mouse breathes 1.5 liters per kgm. per minute. At identical Ct 's, all other factors being equal, the mouse would inhale 15 times as much agent per kgm. body weight as the man because of this difference in pulmonary ventilation rates alone. Thus, on the basis of species difference in respiratory minute volume, a species variation in $L(Ct)$ 50's could be predicted.

Secondly, on exposure to irritating vapors, nearly all animals (2) exhibit a variable degree of inhibition in respiratory rate and amplitude. Inasmuch as the volume breathed determines the amount of toxic agent inhaled at a given concentration, this individually variable inhibition would tend to produce apparent individual differences in susceptibility. Similarly, species differences in degree of respiratory inhibition during exposure would tend to produce apparent species variations in mortality at a given Ct .

Thirdly, the percentage retention of inhaled agent may vary widely between species as well as among members of a given species, depending possibly on respiratory dynamics or characteristics of the respiratory tract such as diameter, moisture, or pH. These variations provide another source of apparent difference in susceptibility when Ct 's are used in assessing the toxicity of inhaled agents.

For these reasons, the use of Ct for the analysis of individual and species susceptibility to inhaled agents has proved unsatisfactory. Tobias, Patt, and Swift (8) attempted measured dose gassing of goats with phosgene. After several preliminary experiments, the methods employed were found to be inadequate. Subsequently, Tobias and Weston (9) developed an improved method and apparatus

by means of which the dose of inhaled agent retained by unanesthetized animals after exposure can be measured accurately. This method, called dosimetric exposure, furnishes experimentally the values of the parameters of equation (3) (vide supra).

The present report is concerned with the first application of the dosimetric technique in the toxicological and pharmacological evaluation of an inhaled agent. The method and procedure for determining the respiratory changes during exposure, the retained LD_{50} , and the percentage of inhaled agent retained are illustrated by data obtained on 90 unanesthetized dogs dosimetrically exposed to phosgene. To evaluate the influence of concentration on the toxicity of phosgene, separate LD_{50} 's were determined at different concentrations in each of three groups of 30 dogs.

METHODS AND EXPERIMENTAL PROCEDURE. The most probable median lethal dose of phosgene in mgm./kgm. was calculated from the previously reported ten minute $L(Ct)_{50}$'s (2) for dogs. For these calculations, it was assumed that the average pulmonary ventilation would approach normal values during exposures as long as 10 minutes, and that all of the agent inhaled was retained. Then, a few animals were given the calculated dose, and the resulting mortality was observed. Subsequently, progressively higher or lower doses were administered to a sufficient number of animals to permit the plotting of a dosage-mortality curve.

The apparatus and methods for exposure were essentially those described by Tobias and Weston (9). By means of a low resistance system—consisting of a flexible Vinylite nose-piece, glass valves, a glass bead absorber, and a recording spirometer—the amount of agent removed by each animal from a dynamic chamber and the amount exhaled into the absorber were measured. The spirometer records not only the total respiratory output but also the volume of the individual respirations.

In a typical experiment, after the dynamic gassing chamber had been brought to equilibrium at the desired concentration of phosgene, the animal was weighed and placed in a stock with a head restraint. Then, the volume of gas to be administered was estimated from the following: the previously calculated, desired total dose; the known chamber concentration of agent; and an assumed percentage retention. Since the percentage retention observed in an animal breathing through a dead space is a function of the tidal air volume (9), the assumed percentage retention was modified during the exposure on the basis of the average tidal air, roughly approximated from the spirometer record, and the volume to be administered was corrected accordingly (vide infra). When the animal had breathed the required volume, the nose-piece was disconnected from the valves at the end of an expiration. Following the exposure, while the absorber contents were being collected into a volumetric flask by five successive rinsings with 200 cc. of distilled water, another gas sample was drawn from the chamber to check the concentration. Phosgene in the glass bead absorber or in the bubbler used for chamber sampling was estimated as chloride by a modified Pinkhof's potentiometric titration (10).

To check for possible facepiece leakage during the course of an experiment, 11 dogs were prepared as for dosimetric exposure and were permitted to inhale air from one spirometer and to exhale into another through the facepiece, valves, and absorber. These tests revealed that leakage was negligible (mean leakage = $\pm 1.3\%$; Std. Dev. = 2.2%).

The animals studied were mongrels, certified as normal by the Veterinary Section. Diets, consisting of powdered milk, ground horse meat with powdered bone, dried dog food and cod liver oil supplement, and water were permitted *ad libitum* until the period of experimentation. After exposure, the animals were observed for 96 hours. Any deaths occurring beyond this time were not considered directly attributable to the phosgene. To determine the cause of death and to discover any incidental pathology which might have affected

survival, autopsies were performed on all animals dying within the 96 hour observation period.

CALCULATIONS. 1. *Dose retained after exposure.* The product of the average concentration of agent in the chamber, as determined by the chemical analyses of chamber samples, times the total volume breathed during exposure, as measured by the spirometer, gives the amount of agent removed from the chamber. Analysis of the absorber contents after exposure gives an accurate measure of the phosgene exhaled. Therefore, the dose (D) in mgm./kgm. of phosgene retained by the animal is readily calculated from

$$D = \frac{CV - A}{w} \quad (4)$$

where C = average analytical concentration of phosgene in the chamber in mgm./l., V = total volume of gas removed from the chamber in l., A = absorber content in mgm., and w = body weight in kgm.

2. *Median lethal dose.* The LD50 in mgm. of phosgene retained per kgm. of body weight was calculated from the experimental data for each series of 30 dogs by the method developed by Bliss (11) for small numbers of animals. In these calculations, after preliminary grouping to plot the provisional dosage-mortality regression line, each animal was treated as a separate experimental observation. Although for each series the first computed approximation agreed satisfactorily with the graphic provisional regression line, a second computed estimate was made as a final check.

3. *Average concentration during exposure.* The presence of a dead space in the valves and facepiece complicates the calculation of both the actual concentration of agent inhaled and the percentage of inhaled agent retained. This, however, does not affect the determination of the dose retained, which is merely the difference between the amount of agent removed from the chamber and that found in the absorber.

At the end of each expiration, the concentration in the dead space is always somewhat lower than the chamber concentration, the extent of the dilution depending on the concentration of toxic vapor in the expired air and the relationship between the volume of the dead space and the tidal air. Conversely, during inspiration, as gas is drawn from the chamber, the concentration in the dead space is increased by an increment which also will depend on the dead space-tidal air relationship. At the end of inspiration, some of this more concentrated agent is forced into the absorber during the next expiration without coming into contact with the animal. With successive breaths, the average expiratory and inspiratory concentrations in the dead space approach equilibrium values that are equal to the chamber concentration times a factor² which can be expressed as the sum of an infinite series. The mean concentration during inspiration is given by

$$C_{\text{insp.}} = C \left(\frac{1}{2 - a} \right) \quad (5)$$

and the mean concentration during expiration is given by

$$C_{\text{exp.}} = C \left(\frac{1 - a}{2 - a} \right) \quad (6)$$

where C = the chamber concentration of agent, and $a = \frac{x}{x + y}$, when x = the mean tidal air volume during exposure, and y = dead space volume.

Thus, during dosimetric exposure through a valve-facepiece system with an appreciable dead space, the animal is exposed not to the concentration of agent in the chamber but to

²Dr. S. D. Silver derived the expression of this factor as the sum of an infinite series from experimental data and theoretical formulation provided by the authors.

the concentration in the dead space during inspiration. By means of the correction factor given in equation (5), the mean contact concentration or the average concentration to which the animal is actually exposed can be calculated from the mean chamber concentration during exposure. It follows that Ct 's should be calculated as the products of these corrected concentrations times the durations of exposure in minutes.

For example, the correction factor averaged about 69% for the three series of dogs exposed to phosgene in the present experiments. Consequently, animals dosimetrically exposed at chamber concentrations of 2.5, or 10 mgm./l. actually inhaled phosgene at mean contact concentrations of 1.38, 3.45, and 6.90 mgm./l., respectively.

4. *Actual percentage of inhaled agent retained.* The percentage retention of inhaled agent is defined as the quotient of the amount of agent retained by the animal divided by the total amount to which the animal was exposed. When an animal breathes through a facepiece-valve system with appreciable dead space, if it is assumed that the amount of agent removed from the chamber is equal to the amount to which the animal is exposed, then the percentage retention would be given by

$$\alpha_0 = \frac{CV - \text{absorber content}}{CV}$$

where α_0 = observed percentage retention, C = chamber concentration, and V = volume of gas removed from the chamber. However, even if the animal were to retain 100% of the agent to which it was exposed, this observed retention (α_0) would be less than 100%, because the absorber would contain some of the agent present in the dead space at the end of each inspiration. Since the absorber content is equal to the product of the volume exhaled, which is essentially equal to V , the volume inhaled, times the average concentration of phosgene in the exhaled air, which is given by equation (6), the theoretical percentage retention which would be observed when the actual retention is 100% is given by:

$$\alpha = \frac{CV - C \left(\frac{1-a}{2-a} \right) V}{CV} \quad (7)$$

or, after simplifying the equation,

$$\alpha = \frac{1}{2-a} \quad (8)$$

Where, as before, $a = \frac{x}{x+y}$ (vide supra).

Although in this brief derivation the factor $\frac{1}{2-a}$ is developed as the observed percentage retention when the actual retention is 100%, it can be demonstrated that dividing the observed percentage retention by this theoretical maximal percentage retention will give the actual percentage retention for any animal breathing through a valve-facepiece system. The value of the correction factor is easily determined, since the average tidal air during exposure is calculated readily from the spirometer record, and the dead space volume may be estimated for any apparatus.

RESULTS. Representative pertinent data for each animal in the first series are presented in Table I.

1. *Median lethal dose of phosgene (LD_{50}).* The median lethal doses for the three series of dogs, with the corresponding standard errors and statistical ranges for $P = 0.01$, are given in Table II. The LD_{50} 's for normal dogs exposed at the

different concentrations of phosgene are as follows: 1.31 mgm./kgm. (Std. Err. = 0.097 mgm./kgm.) at a mean contact concentration of 1.38 mgm./l. (approx.

TABLE I

Pertinent data for 30 normal, unanesthetized dogs exposed by dosimetric method to phosgene at a chamber concentration of approximately 2 mgm. per liter*

DOGS		PHOSGENE EXPOSURE					PHOSGENE RETENTION					RESPIRATION		SURVIVAL
Sex	Weight	Vol.	Chamber concn.	Time	Cl _t corrected	Total	Dose retained		Per cent retention			Number of breaths	Average tidal air	
									Observed	Correc-tion factor	Actual			
	kgm.	l.	mgm./l.	min.	γ-min./l.	mgm.	mgm.	mgm./kgm.					cc.	hrs.
F	5.4	2.59	1.97	4.3	5,375	5.11	2.45	0.45	47.8	63.5	75.2	66	39	Survived
M	7.0	3.73	1.98	3.8	5,235	7.39	3.56	0.51	48.2	69.5	69.4	56	67	Survived
F	7.0	3.78	2.01	3.9	5,655	7.58	5.24	0.75	69.1	72.2	95.6	44	86	Survived
F	2.8	4.57	2.17	4.6		9.92	2.27	0.80	22.9					Survived
F	4.5	4.55	2.33	10.8	15,630	10.61	3.35	0.86	36.4	62.1	58.6	131	34	ca. 24
M	3.5	4.58	2.16	4.6	6,200	9.89	3.19	0.91	32.3	62.3	51.8	133	34	Survived
F	6.6	6.39	2.33	2.9	4,564	14.88	6.28	0.95	42.1	67.6	62.3	110	58	Survived
F	7.3	11.33	2.09	10.3	13,821	23.70	8.13	1.11	34.3	64.2	53.2	267	42	Survived
F	5.9	6.14	2.25	4.3	6,734	13.81	6.74	1.14	48.8	69.7	70.0	89	69	Survived
M	7.7	10.29	2.14	4.9	7,310	22.00	9.13	1.18	41.5	69.8	59.4	147	70	Survived
M	3.8	4.43	2.33	3.3	4,980	10.31	4.55	1.20	44.1	64.9	67.9	98	45	Survived
F	7.3	10.16	2.10	5.4	7,952	21.35	8.74	1.20	40.9	70.2	58.2	141	72	48
F	6.8	9.14	2.14	4.9	6,459	19.56	8.36	1.23	42.7	73.2	58.3	153	60	12-24
M	8.0	8.20	2.05	3.3	4,960	16.81	10.41	1.30	62.0	73.2	84.6	89	92	Survived
F	4.4	4.45	2.25	6.2	9,290	10.01	5.87	1.34	58.6	66.3	88.4	85	52	Survived
M	8.0	8.24	2.08	3.5	5,360	17.14	10.88	1.36	63.5	73.6	86.2	89	93	72
F	4.8	4.11	2.34	2.9	4,966	9.64	6.59	1.37	68.4	73.4	93.2	45	91	24-44
F	3.0	4.64	2.17	3.4		10.07	4.14	1.38	41.1					Survived
F	7.9	7.94	2.33	3.1	5,483	18.50	11.16	1.41	57.4	74.6	76.9	79	100	24-44
F	5.5	7.09	2.16	12.3	17,630	15.31	7.80	1.42	50.9	66.2	76.9	140	51	Survived
F	5.0	6.83	2.14	2.8	4,150	14.62	7.19	1.44	49.1	69.2	70.9	103	66	24
M	6.3	8.49	2.15	3.7	5,500	18.25	9.38	1.48	51.4	69.2	74.3	129	66	20-23
F	5.5	5.34	2.25	2.6	4,320	12.02	8.33	1.53	69.2	73.8	93.7	56	95	Survived
M	7.5	10.74	2.11	3.2	5,144	22.67	12.41	1.66	54.8	76.3	71.8	91	118	70
M	6.8	8.88	2.16	3.6	5,497	19.18	11.44	1.68	59.7	70.9	84.2	117	76	12-20
F	6.1	8.25	2.13	2.5	3,890	17.57	10.03	1.68	61.4	73.2	83.8	91	91	36-48
F	8.0	11.74	2.10	4.6	7,119	24.68	13.61	1.70	55.2	73.6	75.0	126	93	12-20
M	11.1	13.67	2.10	5.8	9,550	28.70	19.20	1.73	67.0	78.3	85.6	100	137	36
F	4.1	5.63	2.16	2.8	4,140	12.16	7.22	1.76	59.4	68.6	86.6	90	63	Survived
F	8.4	12.33	2.12	5.7	9,190	26.14	16.91	2.02	64.8	76.1	85.2	108	114	12-20

*Chamber concentration × correction factor = mean contact concentration.

† Cl_t × correction factor = corrected Cl_t.

chamber conc. = 2 mgm./l.); 0.98 mgm./kgm. (Std. Err. = 0.115 mgm./kgm.) at a mean contact concentration of 3.45 mgm./l. (approx. chamber conc. = 5 mgm./l.); and 0.80 mgm./kgm. (Std. Err. = 0.135 mgm./kgm.) at a mean contact concentration of 6.90 mgm./l. (approx. chamber conc. = 10 mgm./l.).

The "*t*" values (Table III) obtained by "Student's" test indicate that the difference between the LD₅₀'s at 1.38 mgm./l. and 6.90 mgm./l. ("*t*" = 3.1) is statistically significant, whereas the differences between the LD₅₀'s at 1.38 mgm./l. and 3.45 mgm./l. ("*t*" = 2.2) and at 3.45 mgm./l. and 6.90 mgm./l. ("*t*" = 1.0) are not statistically significant.

2. *Percentage retention.* The data in Table IV establish the validity of the factor used in calculating both the actual percentage of phosgene retained and the

TABLE II

LD₅₀'s, standard errors, and statistical ranges for dogs exposed to several concentrations of phosgene by the dosimetric method

NUMBER OF DOGS	MEAN CONTACT CONC. OF PHOSGENE	LD ₅₀	STANDARD ERROR	RANGE OF LD ₅₀ FOR <i>P</i> = 0.01
	mgm./l.	mgm./kgm.	mgm./kgm.	mgm./kgm.
30	1.38	1.31	0.097	1.09-1.59
30	3.45	0.98	0.115	0.73-1.33
30	6.90	0.80	0.135	0.52-1.25

TABLE III

"t" Values obtained when LD₅₀'s for normal, unanesthetized dogs exposed by the dosimetric method to several concentrations of phosgene are compared

MEAN CONTACT CONCENTRATION	1.38 MGM./L.	3.45 MGM./L.	6.90 MGM./L.
mgm./l.			
1.38	—	2.2	3.1
3.45	2.2	—	1.0
6.90	3.1	1.0	—

$$*t = \frac{m_1 - m_2}{\sqrt{e_1^2 + e_2^2}}$$

where *m* = LD₅₀

e = corresponding Std. Error

Odds against the occurrence by chance of a deviation as great as that indicated by these "t" values

"t" value	Odds
1.0	2.2 to 1
2.2	35 to 1
3.1	516 to 1

average concentration of phosgene inhaled during exposure. In these experiments, to simulate an animal with 100% retention, a glass bead absorber, moistened with alcoholic alkali, or a soda lime tube was connected to the valve inlet in place of the animal, with the chamber, valves, absorber, and spirometer set up as for an actual dosimetric experiment. By a measured number of strokes of a glass hypodermic syringe, a given volume of phosgene-laden air was withdrawn from the chamber, through the valves and the auxiliary absorber, and ejected through the dead space and the low resistance absorber into the spirometer. As in an

animal experiment, the average tidal air was obtained by dividing the total volume recorded by the spirometer by the number of strokes (or "breaths") taken. Since the tidal air and the dead space volume for the system were known, the theoretical percentage retention was calculated from equation (8). An inspection of the table reveals that the correspondence between the theoretical or calculated percentage retention and that actually observed was excellent.

Since the percentage retention did not change significantly within the range of concentrations studied, the data for the three series of animals exposed to different concentrations were combined in calculating the mean percentage retention and the statistical range. The mean actual percentage of inhaled phosgene retained by 90 dogs was 74.0% (Std. Dev. = $\pm 12.1\%$ and Std. Err. = $\pm 1.3\%$). In these experiments no significant correlation could be obtained between the actual percentage retention and the tidal air depth, or minute volume, or body weight, or the concentration of phosgene inhaled.

TABLE IV

Tests of percentage retention observed in valve and absorber system with 100% actual retention

EXP. NUMBER	NUMBER OF "BREATHS"	PERCENTAGE RETENTION "OBSERVED"		DIFFERENCE
		Calculated	Actual	
		%	%	%
1	150	68.4	69.1	+0.7
2	130	76.0	75.2	-0.8
3	150	67.3	65.0	-2.3
4	140	73.1	72.5	-0.6
5	35	81.3	82.4	+1.1
6	15	80.2	79.8	-0.4
7	30	82.2	79.9	-2.3
8	20	83.9	83.4	-0.5

3. *Respiratory change during exposure to phosgene.* During exposure to phosgene, nearly all animals studied exhibited respiratory inhibition of varying degrees. This inhibition was manifested not only by breath-holding but by decreased minute volume early during exposure, even after hypercapnia and anoxia had stimulated the animal to breathe. In a typical experiment, the animal, on exposure, would take a sniff of phosgene, exhale promptly, and then hold its breath (cf. Table V). When the animal finally began to breathe, the respiratory rate was slow, and each breath was shallow; consequently, the minute volume was markedly reduced. Gradually, as the exposure continued, the rate and amplitude of respiration increased, and the minute volume was so accelerated that by the third minute it was equal to or greater than the normal value of 200 cc./kgm./min. for dogs (Table VI). The return of respiratory output towards normal results probably from a combination of progressive hypercapnia and anoxia plus accommodation to the irritant action of phosgene. In the animals exposed at higher chamber concentrations (5 and 10 mgm./l.), the short period of exposure is over before the primary respiratory inhibition has passed. Consequently, the average minute volume is more markedly reduced in these groups (Table VII).

The extreme individual variation in the degree of respiratory inhibition during phosgene exposure should be emphasized (Table VII). This was true at all concentrations. As a result of this variability, the times of exposure to a given concentration required to give equivalent doses of phosgene to individual animals

TABLE V

Number of dogs exhibiting breath-holding for specified periods of time after initial upper respiratory contact with phosgene at mean concentrations of 1.38, 3.45, or 6.90 mgm./l.

MEAN CONTACT CONC. OF PHOSGENE	DURATION OF PERIOD OF BREATH-HOLDING				
	0-15	15-30	30-45	45-60	60-75
mgm./l.	sec.	sec.	sec.	sec.	sec.
6.90	23 (82.1%)	5 (17.9%)	0	0	0
3.45	21 (70.0%)	4 (13.3%)	2 (6.7%)	0	0
1.38	24 (80.0%)	5 (16.7%)	1 (3.3%)	0	0

TABLE VI

Average respiratory minute volume per kgm. observed in two groups of thirty dogs during successive minutes of dosimetric exposure to phosgene

MEAN CONTACT CONC. OF PHOSGENE	MINUTE OF EXPOSURE		
	1st	2nd	3rd
mgm./l.	cc./kgm./min.	cc./kgm./min.	cc./kgm./min.
1.38	129.5	274.3	238.3
6.90	103.5	189.0	—

TABLE VII

Average respiratory minute volume per kgm. in three groups of normal dogs during dosimetric exposure to different concentrations of phosgene

NUMBER OF DOGS	MEAN CONTACT CONC. OF PHOSGENE	AVE. MIN. VOL. DURING EXPOSURE			NORMAL AVERAGE MINUTE VOLUME FOR DOGS
		Mean	Standard deviation	Standard error	
	mgm./l.	cc./kgm./min.	cc./kgm./min.	cc./kgm./min.	cc./kgm./min.
30	1.38	294	118	21	200
30	3.45	143	79	14	
30	6.90	167	103	19	

differed considerably, as inspection of Table I will demonstrate. There were, therefore, wide discrepancies between *Cl*'s and actual doses retained (*vide infra*).

4. *Concentration-time products (Cl's)*. The extensive individual variation in the degree of respiratory inhibition during contact with phosgene, as reflected by the coefficients of variation during exposure, results in frequent discrepancies

between Ct (calculated from corrected mean contact concentrations) and the actual dose of phosgene retained after exposure (cf. Table I). This is particularly clear in the animals exposed to the highest concentrations and accounts, in part, for the reported differences in individual susceptibility when Ct is used as a measure of dose.

With lower concentrations and longer exposure times, the period of maximal respiratory inhibition is short in comparison with the total exposure. Consequently, with lower concentrations and longer exposures the total volume of gas inhaled by the animals is within the limits of normal variation of respiration in dogs, and a better correlation is observed between Ct and dose. This somewhat better correlation holds only when large numbers of animals are included, as the individual variation in normal respiration in unanesthetized animals may be quite large.

5. *Pathology.* On autopsy, the animals dying within the 96 hour observation period exhibited the classical picture of phosgene poisoning as described by Winternitz (12). Since deaths occurring more than 96 hours after exposure to phosgene are probably not due to typical pulmonary edema, the two dogs of the 90 studied which died after 110 and 144 hours, respectively, are listed as surviving.

Discussion. In a general review of the literature, Clark (5) stated that the effects of inhaled toxic gases depend not only on the concentration of vapor and the time of exposure but also on the amount of pulmonary ventilation and the extent of absorption by the respiratory surfaces. In addition, he criticized the formula, $Ct = \text{constant}$, "as an impossible one in the case of drugs acting on biological material because it implies that an infinitely small concentration of drug will produce the selected action in a given time and that, conversely, a sufficiently high concentration will produce an instantaneous effect." He concluded that because of the difficulties in measuring the actual dose of inhaled agent retained, the Ct vs. mortality curve constitutes the only basis for the comparison of the relative toxicity of such agents, and then, only if the rates of detoxification for the groups being compared are not too dissimilar.

Henderson and Haggard (6) noted that "the practice of expressing physiological response to gases and vapors only in terms of the concentration of gas or vapor in the air breathed and the length of the exposure" has obscured the facts that the effects of toxic materials whether inhaled, ingested or injected are determined by the total dosage which accumulates in the body and that the physical properties of the gas as well as the physiological peculiarities of the organism are important in the process of absorption through the lung. Prentiss (4) made similar comments in a monograph.

Bodansky and Ginsburg (3) observed that the absence of a respiratory term in the Haber equation makes this equation of little value in accurately determining the dose of HCN which enters the circulation after exposure. More recently, Hutchens and Lipton (7) surveyed the problem of the toxicity of several volatile substances and summarized the evidence supporting the view that a better experimental procedure than Ct exposure is required for the analysis of individual or species susceptibility to inhaled agents.

The results of this study establish the validity of assessing the toxicity of inhaled agents in terms of retained dose rather than Ct 's. By dosimetric exposure the wide discrepancy between Ct and dose, particularly after short exposures to high concentrations of phosgene, is strikingly demonstrated. Moreover, by this procedure, it has been possible to narrow considerably the range of apparent individual variation in resistance to phosgene within a species and to define the toxicity of phosgene for a given species in terms which eliminate the influence of respiratory differences between not only individual animals but also different species.

In addition, it has been possible to explain satisfactorily the reports that $L(Ct)_{50}$ values for phosgene increase as the concentration is increased and exposure time is decreased (2). Since the dose of phosgene acquired during any exposure depends on the amount inhaled, the marked respiratory inhibition at higher concentrations makes longer periods of exposure and, therefore, greater Ct 's necessary if animals exposed to high concentrations of phosgene are to acquire doses equivalent to those acquired at a given Ct at low concentrations.

The determination of the percentage of agent retained after inhalation permits the calculation of valuable information from some of the older, accurate chamber exposure data. By correcting for percentage retention and by assuming that, at exposures of ten minutes or longer, the average respiratory output is equal to the mean normal value for the appropriate species, the LD_{50} in mgm./kgm. body weight can be estimated from the $L(Ct)_{50}$'s.

That the statistical ranges of the LD_{50} 's for phosgene in the dog are greater than those generally reported for systemic poisons like cyanide is not surprising. There are many, not too-well-defined, factors determining susceptibility of an agent like phosgene, the toxic action of which probably is limited to part of one organ, the lung. Moreover, little is known of the relationship between these probable factors—the area of lung surface affected by phosgene, the ratio of the respiratory epithelial surface to the upper respiratory tract, the rate and depth of respiration, etc.—and the body weight, the unit in terms of which the dose of phosgene is expressed. In this connection, it is interesting to note that there is a wide variation between members of a given species as regards the relationship of the weight of a given organ to the body weight. Brown, Pearce, and Van Allen (13) reported that the coefficient of variation for the ratio between organ weight and body weight in 643 laboratory rabbits varied from 11.9% for the heart to 61.39% for the thyroid. Unfortunately, they cited no figures for the lung, but these data indicate the great individual variation in the ratio of organ mass to body weight, in general.

Toxicity of phosgene is dependent on a number of variables which may or may not be proportional to the body weight. Therefore, eliminating only two variables, the volume of agent inhaled in a given time and the per cent of inhaled agent retained, and expressing the resulting dose in mgm./kgm. does not necessarily eliminate all the factors in individual susceptibility which make for a less steep dosage-mortality curve. Inasmuch as the pharmacological effect of any material is best expressed as the amount per unit of affected tissue, the LD_{50} of a

poison like phosgene with a localized site of action, the lung, probably should be expressed in terms of some functional or anatomical pulmonary unit such as lung surface area. However, the present inability to define such a unit makes it necessary to report the median lethal dose in terms of the body weight.

The apparent, progressive increase in the toxicity of phosgene for dogs with the increase in concentration hitherto has not been recognized. The interpretation of such a change in toxicity with concentration must be hypothetical at this stage, but several factors probably are involved, including minimal tolerated dose of phosgene per minute and per cent removed in the upper respiratory tract.

These experiments have provided new lines of approach for the pharmacological and toxicological analysis of inhaled substances and have opened the way for the re-evaluation of the prophylactic and active therapy of phosgene poisoning. A reproducible dosage-mortality curve has been established, from which the retained median lethal dose has been defined in mgm./kgm. with the statistical range for the usual fiducial limits. Now, it is possible to compare with accuracy the relative toxicity of phosgene in different species, using the "*t*" test to evaluate the statistical significance of any differences in the LD₅₀'s of the species studied. In addition, the influence of different experimental or therapeutic procedures on the toxicity of phosgene may be assayed in any species, using the parameters of the dosage-mortality curve in determining the statistical significance of any changes observed (14). Such experiments will be reported in subsequent papers.

SUMMARY

1. The theoretical basis for biologically assaying inhaled materials in terms of retained dose rather than concentration-time products was discussed.

2. The methods and procedures for determining the retained dose of an inhaled substance and for calculating the actual percentage of agent retained after inhalation were presented.

3. The principles and application of the dosimetric method were illustrated by experiments on 3 groups of 30 unanesthetized dogs in which the median lethal dose, percentage retention of inhaled agent, and respiratory changes during dosimetric exposure to 3 different concentrations of phosgene were determined.

4. The retained median lethal doses of phosgene for dogs exposed to phosgene at mean contact concentrations of 1.38, 3.45, and 6.90 mgm./l. were as follows: 1.31 mgm./kgm. (S.E. = 0.097 mgm./kgm.), 0.98 mgm./kgm. (S.E. = 0.115 mgm./kgm.), and 0.80 mgm./kgm. (S.E. = 0.135 mgm./kgm.), respectively. The increase in toxicity when the phosgene concentration was increased from 1.38 mgm./l. to 6.90 mgm./l. is statistically significant ("*t*" = 3.1) and requires further experimental analysis.

5. The mean percentage of inhaled phosgene retained by the 90 dogs was 74.0% (Std. Dev. = $\pm 12.1\%$; S.E. = $\pm 1.3\%$). In this series no significant correlation could be made between percentage retention and tidal-air depth, or respiratory minute volume, or concentration of phosgene inhaled.

6. During the first two minutes of exposure, all animals exhibited respiratory inhibition which varied considerably in different individuals, was greatest at the

highest concentrations, and was manifested by a brief period of breath-holding followed by a longer period of shallow, slow breathing. By the third minute, the respiratory minute volume was equal to or slightly greater than normal, probably as a result of hypercapnia, anoxia, and accommodation to the irritant vapors.

7. Wide discrepancies were demonstrated between concentration-time products (Ct 's) and the actual retained dose, particularly in the animals exhibiting the greatest respiratory inhibition during exposure. As exposure time was increased, however, there was a somewhat more uniform relation between Ct and retained dose.

8. The experimental data reported are considered to establish the validity of biologically assessing inhaled substances in terms of retained dose rather than Ct exposures. The technique of dosimetric exposure should permit re-evaluation of certain toxicological and therapeutic aspects of poisoning by inhaled noxious agents.

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THE ASSAY OF THE RENOTROPHIC ACTIVITY OF THE ANTERIOR PITUITARY

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It has been reported by Selye and co-workers (1, 2) that administration of a crude anterior beef pituitary preparation can cause preferential growth of kidney tissue. This is accompanied by an increase in kidney function (3). These same crude pituitary preparations in salt-treated, partially nephrectomized rats, can induce "nephrosclerosis", a type of kidney damage characterized by dilation of tubules, formation of hyalin casts, hyalinization of glomeruli and arteriolar necrosis (4). It is not known whether the enlargement and damage are due to the same or different principles or whether either effect is associated with a known pituitary hormone, a combination of known hormones, or to a new principle. To investigate this problem, a method for assaying the nephrosclerotic activity of pituitary preparations has been developed (5). In the following report, a method for the bioassay of the renotrophic effect is described together with some of the factors which influence it.

EXPERIMENTAL. The use of the hypophysectomized rat as a test animal was investigated. Male albino rats, 85 to 105 grams in body weight were hypophysectomized and one day later, administration of a lyophilized anterior pituitary (L.A.P.) suspension was commenced. Doses ranged from 2.5 to 20 mg. per day, and were given in two daily subcutaneous injections of 0.2 cc. each for 10 days. At autopsy, kidneys were fixed in Suza, and 24 hours later, changed to a 4% formalin solution before being weighed. Kidney weights were expressed in terms of grams per 100 grams body weight, and milligrams per 100 square centimetres body surface area.¹ No significant renotrophic effect was seen with any dose administered when expressed in terms of body weight. When expressed in terms of body area, even 2.5 mg. per day produced a significant, though probably minimal response. The relative kidney weight per 100 cm.² surface area for the controls was 455 ± 16 mg. and for those given 2.5 mg. L.A.P. daily, 508 ± 15 mg. However, an 80% mortality with the lowest dose as compared with a 10% mortality in controls precluded the use of this method for the assay of crude pituitary preparations.

On the other hand, the hypophysectomized rat may be employed to assay more purified preparations. This is clear from results obtained with the fol-

¹MacKay and MacKay (6) have⁶ pointed out that whereas the kidney to body weight ratio decreases with increasing body weight, the kidney weight to body surface ratio remains constant over a very wide range. To calculate body surface, the constant 9.1, suggested by Benedict (7), was employed in the formula: $S = K \times W^{\frac{1}{2}}$ where S is the surface area in square centimetres, K, the constant and W, the body weight in grams.

lowing two extracts. As shown in Table I, the mortality in the experimental groups was no greater than in the control. The method of assay used was the same as that described above. Extract No. I was prepared so as to selectively retain the thyrotrophic principle and eliminate the somato- and adrenotrophic.

100 grams of L.A.P. were ground with sand and extracted twice with 500 cc. 1% NaCl at pH 8.5-9.0. The pH of the combined extracts was adjusted to 7.5, the mixture centrifuged, and the supernatant brought to pH 5.4. The supernatant after centrifuging was brought to pH 4.8, centrifuged, the supernatant again retained, brought to pH 4.1 and centrifuged. Acetone to 40% was added to the supernatant obtained. After standing over night, the supernatant was separated and brought to 80% acetone. After standing over night again,

TABLE I

Bioassay of purified pituitary preparations in hypophysectomized rats

GROUP	EXTRACT NO.	BODY WEIGHT		NO. OF RATS		KIDNEY		t ₁ *	t ₂ *
		Initial	Final	Initial	Final	%/B.W.	%/S		
1	1	82	78	7	4	0.89 ± 0.05	419 ± 23	2.81	3.27
2		82	73	9	7	1.07 ± 0.04	494 ± 12		
3	2	92	91	4	3	0.72	379 ± 21		0.47
4		88	122	4	4	0.73	395 ± 27		

$$* t = \frac{\text{difference between means}}{\text{S. E. difference}}$$

$$\text{S.E. difference} = \sqrt{\text{S.E.}_1^2 + \text{S.E.}_2^2}$$

$$\text{S.E.} = \sqrt{\frac{\sum(X - \bar{X})^2}{n(n-1)}}$$

Where X is the observed value, \bar{X} , the mean, and n , the number of animals. A value of 2.228 for the t of a mean difference between groups of 6 animals represents a P of 0.05, a t value of 2.764, a P of 0.02, and a t of 3.169, a P of 0.01.

t_1 is the t of the difference between % kidney to body weight ratios in injected and control groups.

t_2 is the t of the difference between % kidney weight to body surface area ratios in injected and control groups.

the precipitate was collected by centrifugation, suspended in 100 cc. water and dialyzed for 72 hours. The precipitate was removed by centrifuging and the supernatant tested by injecting 0.17 cc. twice daily subcutaneously (4.16 mg. protein per day). This dose was just adequate to produce a significant increase in thyroid weight in the test rats employed but no adreno- or somatotrophic effect (control average thyroid weight, 7 ± 0.9 mg., experimental, 10 ± 0.9 mg.).

The second extract was prepared so as to selectively retain somatotrophic activity and eliminate thyrotrophic.

200 grams of L.A.P., washed with acetone to remove fat, were extracted with 6 litres of Ca(OH)_2 solution at pH 11.5 for 24 hours. The pH was lowered to 8.7 by passing CO_2 into the solution. After standing 24 hours, the insoluble material was removed by centrifugation. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 2.0 M. The precipitate which separated on standing was suspended in 2 litres of water and the solution made 0.6 M with ammonium sulphate. The precipitate was discarded and the supernatant

brought to 2.0 M. The precipitate formed was suspended in 500 cc. water and dialyzed until salt free. The pH of the dialyzed material was adjusted to 6.7, the precipitate collected and dissolved in 300 cc. water by adding 1.0 M HCl to pH 4.0. Saturated NaCl solution was added to 0.1 M. The supernatant was separated and brought to 5.0 M with solid NaCl. The precipitate formed was dissolved in 100 cc. water, dialyzed until salt free. The precipitates formed by adjusting the pH of this solution to 5.7 and 8.7 were separated off. The clear supernatant was made 1.65 M with ammonium sulphate at pH 7. The precipitate obtained was dissolved in 100 cc. water at pH 8.2 and then adjusted to pH 7.5. The supernatant was tested by injecting 0.15 cc. twice daily subcutaneously (0.68 mg. protein per day). This dose produced no significant thyrotrophic response (control average thyroid weight, 7 ± 1 mg., experimental, 9 ± 1), but a very marked somato- and adrenotrophic response. The body growth stimulation was equivalent to that seen with *ca* 6.7 mg. L.A.P. per day and the adrenal enlargement was greater than that seen with 15 mg. of L.A.P. daily in similar test rats (average adrenal wt. of controls, 10 ± 0.9 , of experimentals with 15 mg. L.A.P. daily, 13 ± 0.3 mg., with Extract No. 2, 16 ± 1.3).

In Table I are shown the renotrophic effects seen with these two extracts. Extract No. 1 selectively stimulated kidney growth without influencing body growth, while Extract No. 2 stimulated body growth but did not selectively increase kidney growth. It would appear therefore that the renotrophic effect of anterior pituitary is not directly associated with the growth hormone. Extract No. 1 contained thyrotrophic hormone which is known to be renotrophic (8, 9). However, the renotrophic effect of L.A.P. can be elicited in the absence of the thyroid (10). Thus it cannot be due simply to the thyrotrophic activity. No physical chemical analysis of the extracts was made so the number of proteins present and their amounts is not known. It is quite possible the renotrophic effect may be due to a combination of known principles but it is certainly not associated simply with thyrotrophic or growth hormones.

Although this hypophysectomized rat method proved useful for assaying highly purified preparations, because of the high mortality another method had to be developed to follow the activity during extraction. Attention was turned to the use of fasted rats. It was thought that L.A.P. in the fasted rat would maintain or augment kidney tissue at the expense of other body tissues.

A comparison was made between the renotrophic activity of L.A.P. in groups of 8 male rats fasted and treated, and fed and treated for 1, 2, 3, and 4 days. No preferential stimulation of kidney growth was seen in less than 3 days in either fasted or fed immature animals injected with 10 mg. of L.A.P. twice daily. On the 3rd day, a statistically significant renotrophic effect was produced in fasted rats when expressed in terms of body surface. The average relative kidney weight of fasted controls was 470 ± 29 mg. per 100 cm² body surface, and of fasted treated, 568 ± 25 . By the fourth day a significant effect was induced in both fasted and fed rats expressed both in terms of body weight and body surface. The average relative kidney weight of fed controls was 1.01 ± 0.03 g. per 100 g. body weight or 501 ± 9 mg. per 100 cm.² surface, while that of treated and fed rats was 1.17 ± 0.03 g. per 100 g. body weight or 588 ± 10 mg. per 100 cm.² surface. For fasted controls and fasted, treated animals, the respective averages were: 1.19 ± 0.03 g., or 507 ± 6 mg. and 1.49 ± 0.07 g., or 641 ± 33 mg.

Since the renotrophic effect in this experiment was almost if not quite as marked in the fed as in the fasted animals, attention was turned to the use of normal, fed rats for the assay of this activity.

Male hooded rats, weighing initially 40 to 50 grams were employed throughout, except in the last three experiments in which rats 50 to 60 grams, were used. They were fed "Purina Fox Chow"² *ad lib.* L.A.P. was suspended in 10% alcohol and administered in two, 0.2 cc. subcutaneous injections daily. Table II summarizes the results obtained with various doses given for 5, 7, 10 and 14 days.

Although 20 mg. of L.A.P. administered daily for four days produced a significant renotrophic response in the experiments just described, 10 mg. daily for 5 days did not (Table II, experiment 1). Since the concentration of protein handled would have to be high in order to administer purified extracts in doses equivalent to 20 mg. daily in suitable volumes, it was decided to lengthen the duration of treatment with the expectation that a smaller daily dose would be required.

In experiments 2 and 7, the same lot of L.A.P. in doses of 2.5, 5 and 10 mg. daily, were administered for 7 and 10 days. A statistically significant response was obtained with 5 mg. daily for 10 but not 7 days when expressed in terms both of body weight and body surface. A significant response occurred also with injection of 10 mg. daily for both 7 and 10 days when expressed in terms of body surface. Experiments 3 and 4, in which the same batch of L.A.P. was used as was employed in experiments 2 and 7, show that 6 to 8 mg. of L.A.P. were required to obtain a significant renotrophic effect in 7 days, in terms of body surface. The author cannot explain the lack of response seen in group 5, experiment 4, but this is the only group in the whole series which behaved in an irregular manner.

From these four experiments, and a perusal of the last two columns of Table II it is clear that the renotrophic activity should be expressed in terms of body surface area. Expressed in terms of body weight, the response is very irregular and a higher dose is required—though even with a dose of 20 mg. per day an effect may not be induced (experiment 9).

Experiment 4 was performed to investigate whether testis extirpation in these immature animals would materially influence the response. It was possible that the L.A.P. might contain enough gonadotrophin to stimulate the testis to release testoids which, in turn, have a well recognized renotrophic effect. Groups 2, 4 and 6 were castrated the day before injections were commenced. From a comparison of the responses observed it is clear that castration has no effect on the sensitivity of the method.

Although partial nephrectomy was found to increase sensitivity to the renotrophic effect of L.A.P. slightly, this intervention also sensitizes the rat to the nephrosclerotic effect. For this reason it was deemed inadvisable to employ rats so operated for the renotrophic assay.

²A cubed food prepared by Ralston Purina Company Ltd, Montreal.

TABLE II
Bioassay of L.A.P. in normal male rats

EXPERIMENT NO.	GROUP NO.	DAILY DOSE OF L.A.P.	NO. OF RATS	FINAL BODY WEIGHT	AV. KIDNEY WEIGHT		t ₁ [°]	t ₂ [°]
					%/B.W.	%/B.S.		
5 days treatment								
1	1	0	6	60	1.36 ± .03	589 ± 18	0.55	1.37
	2	8	6	72	1.38 ± .02	622 ± 15		
7 days treatment								
2	1	0	6	70	1.28 ± .03	581 ± 15	-2.50 .84 2.00	-1.01 1.17 3.31
	2	2.5	6	78	1.19 ± .02	561 ± 11		
	3	5	6	79	1.36 ± .09	639 ± 29		
	4	10	6	87	1.38 ± .04	668 ± 22		
3	1	0	6	67	1.27 ± .03	566 ± 12	0.50 1.19	2.06 2.74
	2	4	6	74	1.29 ± .03	597 ± 9		
	3	8	6	77	1.32 ± .03	618 ± 15		
4	1	0	6	70	1.23 ± .06	580 ± 22	1.49 1.80 .60 3.20	2.76 4.88 1.78 5.42
	2	0	7	66	1.27 ± .03	565 ± 15		
	3	6	7	87	1.33 ± .03	652 ± 14		
	4	6	6	87	1.36 ± .04	665 ± 14		
	5	10	6	90	1.27 ± .03	630 ± 19		
	6	10	7	84	1.43 ± .04	692 ± 18		
5	1	0	6	62	1.23 ± .02	536 ± 11	3.36	5.43
	2	8	6	80	1.38 ± .04	650 ± 18		
6	1	0	6	68	1.23 ± .03	553 ± 13	3.80	4.57
	2	8	6	85	1.35 ± .01	649 ± 17		
10 days treatment								
7	1	0	6	70	1.20 ± .03	542 ± 13	0.87 7.20 4.30	4.84 4.30
	2	2.5	6	74	1.27 ± .03	585 ± 20		
	3	5	6	77	1.36 ± .04	638 ± 15		
	4	10	6	80	1.37 ± .03	652 ± 22		
8	1	0	8	76	1.18 ± .04	550 ± 16	-1.60 0.00 1.78	0.80 2.88 5.52
	2	8	9	100	1.11 ± .03	567 ± 14		
	3	10	10	97	1.18 ± .01	599 ± 7		
	4	12	10	99	1.26 ± .02	644 ± 5		
9	1	0	8	67	1.25 ± .05	558 ± 22	1.85	4.84
	2	20	8	92	1.35 ± .02	679 ± 12		

* See footnote to Table I.

TABLE II—*Concluded*

EXPERIMENT NO.	GROUP NO.	DAILY DOSE OF L.A.P.	NO. OF RATS	FINAL BODY WEIGHT	AV. KIDNEY WEIGHT		t ₁ *	t ₂ *
					%/B.W.	%/B S.		
14 days treatment								
10	1	0	6	99	1.13 ± .01	574 ± 12	2.84	4.42
	2	5	6	123	1.22 ± .03	669 ± 15		
11	1	0	6	98	1.10 ± .03	556 ± 9	1.20	3.60
	2	8	6	128	1.16 ± .04	662 ± 28		
12	1	0	6	116	1.06 ± .01	570 ± 6	1.25	3.52
	2	10	6	145	1.10 ± .03	637 ± 18		
13	1	0	6	120	1.06 ± .02	574 ± 14	1.56	2.40
	2	10	6	132	1.13 ± .04	634 ± 21		
14	1	0	6	115	1.11 ± .02	595 ± 14	.89	2.58
	2	10	6	141	1.16 ± .04	669 ± 25		

When the response to a particular dose in 7 days is compared with that seen to the same dose in 14 days, it is apparent that the full effect has taken place in 7 days. There is no indication in these experiments of how long such a plateau is maintained, but from other experiments performed in this laboratory it is clear that eventually the animals become refractory.

Experiments 7, 8 and 9 show that increasing the dose beyond 10 to 12 mg. does not increase the effect.

Experiments 5, 6, 13 and 14 were performed to show how constant a response could be obtained with a given dose of the same lot of L.A.P. (but different from the one assayed above) tested at different times for 7 and 14 days. It is clear that very consistent effects were seen.

If the responses observed in experiments 2, 7 and 8 are plotted against the *logs* of the doses, it will be seen, however, that the slopes of the curves obtained are somewhat different. It is possible that the same parts of the curves have not been measured. A constant slope was not essential since we wished to determine only whether the renotrophic activity of a certain extract passed into the precipitate or the supernatant under given conditions. For this purpose, the method has proved quite adequate.

It has been shown that in rats sensitized to the nephrosclerotic activity of L.A.P., changes in the composition of the diet markedly influence the size of the kidney and the incidence of lesions (11). The following experiments demonstrate that the renotrophic effect in not-sensitized animals is also markedly effected by the diet.

Each experiment was performed on four groups of 6 hooded male rats 50 to 60 grams in body weight. The first and second groups were fed ground purina fox

chow and the third and fourth, a diet containing 80% ground purina and 20% casein, cornstarch or mazola oil. The rats of the second and fourth groups of each experiment were injected subcutaneously with 10 mg. of L.A.P. twice daily for 14 days. The final body, and relative kidney weights are given with the details of procedure in Table III.

With this short period of treatment, the relative kidney size of control animals was not significantly influenced by the changed composition of the diet. In each experiment, a consistent renotropic response to L.A.P. was induced in the rats fed pure purina. This response was suggestively, but not significantly increased in rats fed the casein enriched diet. It was completely inhibited in rats fed either the starch or fat enriched diets.

TABLE III
Effect of quality of diet on renotropic response

EXPERIMENT NO.	GROUP NO.	DIET	DOSE OF L.A.P. <i>mg. per day</i>	FINAL BODY WEIGHT	% KIDNEY WT. BODY SURFACE AREA
1	1	100% purina	0	115 \pm 5.6	595 \pm 14
	2	100% purina	10	141 \pm 3.2	669 \pm 25
	3	80% purina, 20% casein	0	106 \pm 4.8	584 \pm 20
	4	80% purina, 20% casein	10	132 \pm 7.1	718 \pm 34
2	1	100% purina	0	120 \pm 3.1	574 \pm 14
	2	100% purina	10	132 \pm 4.6	634 \pm 21
	3	80% purina, 20% starch	0	119 \pm 3.8	569 \pm 13
	4	80% purina, 20% starch	10	146 \pm 2.6	561 \pm 18
3	1	100% purina	0	116 \pm 3.8	570 \pm 6
	2	100% purina	10	145 \pm 3.8	637 \pm 18
	3	80% purina, 20% oil	0	97 \pm 7.0	532 \pm 18
	4	80% purina, 20% oil	10	115 \pm 3.9	508 \pm 11

The lack of response seen in rats fed the increased fat diet cannot be due to the relatively smaller amount of food eaten since it can be elicited in fasted rats, as shown above, and in partially starved rats, as indicated in the following experiment. Twenty female albino rats, weighing 45 to 65 grams, were partially nephrectomized. The day after the operation, ten of these were given purina *ad lib.* and the other ten were pair fed for 28 days. During this period, 12 mg. of L.A.P., were administered twice daily to the latter group. The same amount of casein was given in the same manner to the controls. Both drank tap water throughout. At autopsy, the average final body weight of the control group was 129 grams, and of the L.A.P. treated group, 136 grams. The relative kidney weights were 424 ± 16 mg., and 483 ± 12 mg. respectively. There is only one chance in 50 that the difference is not significant. The kidneys were sectioned histologically and found completely free of nephrosclerosis. Thus the renotropic effect can be elicited in rats whose food intake is limited to the appetite of untreated rats.

SUMMARY AND CONCLUSIONS

The renotrophic activity of the anterior pituitary may be assayed in normal immature male rats 40 to 50 grams in body weight, fed purina fox chow, and injected subcutaneously twice daily for 7 days with the pituitary preparation in suspension or solution, if the kidney weights are expressed in terms of the body surface area, and quantitative comparisons of activities are made between groups of a single experiment performed at one time.

Hypophysectomized rats may be employed satisfactorily to test highly purified, but not crude preparations, due to the toxicity of the latter. In this test animal a potent thyrotrophic extract was found renotrophic, while a highly somato- and adrenotrophic preparation produced no preferential kidney enlargement. The renotrophic response is not associated simply with thyroid stimulation, however, since it can be elicited in thyroidectomized rats (10).

A significant response is obtained with large doses of lyophilized anterior pituitary (L.A.P.) administered daily to rats fasted three or four days, but it is not greater than is seen in normal rats treated 7 days with smaller daily doses.

Castration does not influence the response.

Maintaining the total food intake and thus the protein intake, of L.A.P. treated and fed rats to that of untreated controls does not inhibit the appearance of the renotrophic effect.

Feeding a 20% starch or mazola oil, and 80% purina diet to rats treated with 20 mg. of L.A.P. daily for 14 days inhibited the enlargement of the kidney seen consistently in similarly treated rats fed purina only.

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OXIDATION OF PHENETHYLAMINE DERIVATIVES BY AMINE OXIDASE

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Since the discovery of amine oxidase in liver (1) a number of workers have attempted to determine the physiological significance of this enzyme system. Hare (1) believed that it might function to detoxify tyramine absorbed from the intestine. The demonstration of the deamination of epinephrine and other amines by amine oxidase *in vitro* (2) led Gaddum and Kwiatkowski (3) to physiological studies which indicated that amine oxidase was capable of destroying the pharmacological activity of epinephrine and that potentiation of its activity by ephedrine was due to the protection of epinephrine against destruction by amine oxidase. From studies of dosage-duration curves on physiological systems, Clark and Raventós (4) suggested that tissue oxidases limited the duration of the pharmacological effects of epinephrine and tyramine. Their finding that tyramine was inactivated about five times as rapidly as epinephrine was correlated with the conclusions of Blaschko *et al.* (2) that tyramine was oxidized more rapidly than epinephrine by amine oxidase. Also, Fuhrman *et al.* (5) found that the *in vitro* rate of oxidation of epinephrine by amine oxidase was dependent on temperature and that the *in vivo* rate of inactivation, as measured by the duration of action on the nictitating membrane, similarly was dependent on body temperature, particularly liver temperature. This led them to believe that the physiological inactivation of epinephrine was an enzymatic process although not necessarily limited to amine oxidase. However, it was believed by Richter and Tingey (6) and Kohn (7) that the oxidation of epinephrine at physiological concentrations by amine oxidase was too slow to be of physiological significance. Alles *et al.* (8) held a similar view but calculated that tyramine inactivation could be accounted for by amine oxidase activity. Snyder and co-workers (9) observed that the urinary excretion of phenethylamine derivatives that were attacked by amine oxidase was very low.

The possibility that amine oxidase may inactivate circulating amines prompted an attempt to correlate the relative ease of oxidation of a series of phenethylamine derivatives with the pharmacological properties of the amines. There was available for this purpose a series of closely related phenethylamine derivatives considerably more extensive than those studied previously. The series included the unsubstituted primary, secondary and tertiary phenethyl amines and analogous derivatives having hydroxy and methoxy groups substituted in the molecule. A few quaternary salts were also included.

METHODS. The compounds used as substrates were synthesized in these laboratories by J. S. Buck and associates (10).

The rates of oxidation of amines were determined in the Warburg apparatus using homo-

genized liver Brei prepared according to the technique described by Beyer (11). A weighed sample of liver was homogenized in two volumes of distilled water and two volumes of 0.2 M phosphate, pH 7.2, were added. The Brei was strained through muslin and used immediately or kept at 0°C. between experiments. Each reaction vessel contained 1.4 ml. of Brei, 0.5 ml. of 0.05 M amine hydrochloride and 0.1 ml. of 0.1 M sodium cyanide in a total volume of 2.0 ml. The center cup contained filter paper saturated with 10% potassium hydroxide. The rate determination was carried out at 38°C. with shaking at the rate of 120 oscillations per minute. The oxygen uptake was read at 5 minute intervals, and the average rate for the first 20 minutes was calculated for each compound. The rate of oxygen uptake of the Brei alone was determined with each set of experiments, and the rate of autoxidation of the phenolic amines was determined in the presence of boiled Brei. The blank values

TABLE 1

Comparative rates of oxidation of phenethylamine derivatives by amine oxidase in liver brei

PHENETHYLAMINE HYDROCHLORIDES SUBSTITUENT GROUPS	PRIMARY AMINES			SECONDARY AMINES			TERTIARY AMINES		
	Com- pound no.	Guinea pig	Cat	Com- pound no.	Guinea pig	Cat	Com- pound no.	Guinea pig	Cat
		%	%		%	%		%	%
4-Hydroxy.....	26	100	100	32	92 ± 3	98 ± 5	687	5 ± 1	40 ± 3
3-Hydroxy.....	694	69 ± 3	70 ± 6	31	121 ± 2	83 ± 2	686	18 ± 2	50 ± 5
2-Hydroxy.....	693	16 ± 3	64 ± 6	30	38 ± 4	63 ± 4	685	2 ± 1	30 ± 2
3,4-Dihydroxy.....	697	122 ± 2	91 ± 5	22	113 ± 3	82 ± 4	689	19 ± 1	29 ± 1
3,4 - Dihydroxy - β - hydroxy.....				21	40 ± 1	18 ± 3			
Unsubstituted.....	25	19 ± 3	25 ± 2	45	63 ± 7	85 ± 4	672	5 ± 2	16 ± 3
4-Methoxy.....	669	7 ± 4	39 ± 6	29	23 ± 1	83 ± 5	675	7 ± 3	24 ± 4
3-Methoxy.....	668	31 ± 3	21 ± 5	28	65 ± 3	62 ± 5	674	11 ± 3	16 ± 4
2-Methoxy.....	667	94 ± 4	79 ± 5	27	93 ± 3	68 ± 3	673	12 ± 4	45 ± 4
2,3-Dimethoxy.....	670	73 ± 5	56 ± 2	33	87 ± 9	63 ± 3	676	14 ± 2	31 ± 5
3,4-Dimethoxy.....	671	10 ± 4	25 ± 4	36	12 ± 5	48 ± 1	677	8 ± 3	9 ± 1
2,5-Dimethoxy.....	819	8 ± 4	25 ± 4	35	33 ± 5	29 ± 4	821	16 ± 3	19 ± 4
2,5 - Dimethoxy - β - hydroxy.....	831	5 ± 1	10 ± 2	832	7 ± 2	18 ± 3	833	4 ± 1	12 ± 6

were subtracted in the calculation of the rate of oxidation of the amines. The rate of oxidation of tyramine in the presence of liver Brei was determined in each series of experiments. The rates for the other amines were calculated as per cent of the tyramine rate.

RESULTS. The mean rate for guinea pig liver was $6.0 \pm 0.2 \mu\text{l.}$ per minute and for cat liver $3.7 \pm 0.3 \mu\text{l.}$ These values confirm the species differences noted by Alles *et al.* (8).

The data in the table are the means of the relative percentage rates (per cent of tyramine) obtained with at least three different livers.

The type of substitution on the nitrogen had a dominant effect on the rates of oxidation. In general, the tertiary amines were oxidized less rapidly than the primary and secondary amines and the quaternary salts were not oxidized at all. Although no consistent generalizations could be made as to the relative rates of oxidation of primary as compared with secondary amines, a majority of the secondary amines had equal or greater rates than their primary analogs.

The position of the substituents on the benzene ring likewise markedly influenced the relative rates of oxidation. The 3-hydroxy and 4-hydroxyphenethyl amines were more rapidly oxidized than the 2-hydroxy derivatives. The 2-methoxyphenethylamines were more rapidly oxidized than the 3-methoxy and 4-methoxy derivatives. Among the dimethoxy derivatives, the 2, 3-dimethoxy phenethylamines had the most rapid rates.

The substitution of the hydroxy group in the β -position decreased the rate of oxidation. Thus, epinephrine had a slower rate than its analog, epinine, and the 2,5-dimethoxy- β -hydroxyphenethylamines had slower rates than their analogs having the β -carbon unsubstituted.

Comparison of the analogous hydroxy and methoxy derivatives revealed that the majority of the hydroxy compounds were oxidized more rapidly than the corresponding methoxy compounds; however, the 2-hydroxy phenethylamines were less rapidly oxidized than the 2-methoxy derivatives.

Species differences in the relative order of oxidation were frequently large. Thus, the tertiary amines were usually more rapidly oxidized by the cat liver than by guinea pig liver, while with the primary and secondary amines no consistent trends were apparent. Whereas tyramine was the most rapidly oxidized compound of the series using cat liver, several phenolic compounds were oxidized more rapidly than tyramine when the guinea pig liver was used.

Discussion. The attempt was made in the present work to arrive at generalizations in the relationship of the chemical structure and the rates of oxidation of an extensive series of closely related phenethylamine derivatives in order to compare the ease of oxidation of the amines with their pharmacological properties. The results obtained with this fairly complete series of substituted primary, secondary and tertiary amine analogs in general confirm and extend to new types of compounds the conclusions reached by previous workers (2, 8, 9, 11, 12) who measured the rates of oxidation of some of the above amines as well as of other types of amines. As described in the results above, all of the amines are oxidized, the rates depending upon the substitutions in the phenethylamine nucleus as well as upon the animal species from which the enzyme preparation was obtained. Although Beyer (11) believed that tertiary amines are not oxidized, the present results with 12 tertiary amines confirm the findings of previous workers (2, 8, 9) that they are oxidized, the rates depending upon the constituents in the ring and upon the species. While the guinea pig liver oxidizes them very slowly, the cat liver oxidizes them almost half as rapidly as their primary and secondary analogs. In excretion studies in the rat, Synder *et al.* found that a tertiary amine was destroyed in the body.

It is of interest that the 2,5-dimethoxy derivatives (not previously studied) are oxidized at a slow rate. These compounds also have been found to inhibit the oxidation of tyramine by amine oxidase (unpublished experiments) probably by competition for the available enzyme, just as phenylisopropylamines of the ephedrine-type inhibit the enzyme. These compounds are long-acting pressors of the ephedrine-type (unpublished experiments). The long duration of their action may be related to their ability to inhibit amine oxidase even though they

are slowly oxidized by the enzyme. In this respect their action may be analogous to that of ephedrine and similar compounds, the long action of which is believed due to their inhibiting action on the enzymes which destroy epinephrine (3).

It was believed that one might obtain some insight into the role of amine oxidase in the physiological inactivation of sympathomimetic amines by comparing their rates of oxidation by amine oxidase with their pharmacological activities. However, the relative order of oxidizability by amine oxidase did not parallel closely the relative order of the intensities and durations of the pressor effects of the amines (13, 14). Also no close correlation of the ease of oxidation of the amines with their toxicity values (13) was apparent.

Since no correlation of ease of oxidation of amines with their pharmacological activity has been found, the relative significance of amine oxidase in the inactivation of circulating amines remains obscure. A number of mechanisms for the inactivation of phenolic amines are known. Thus epinephrine and other diphenolic amines are subject to at least three different modes of inactivation. The phenolic groups may be oxidized by the cytochrome oxidase system and the polyphenoloxidase system (15, 16); they may be esterified and excreted as the ester (17); and the side chain may undergo oxidative deamination by amine oxidase (2). Likewise, tyramine and other monophenolic amines may be inactivated by these same mechanisms. On the other hand, methoxy substituted phenethylamines would not be readily subject to oxidation of the ring but would most likely be inactivated by the amine oxidase system. A close relationship between physiological potency and susceptibility to attack by a particular enzyme system could be detected only where that enzyme system was the predominant mechanism of the inactivation and would appear only among a group of compounds in which the structural differences were not marked.

The author is indebted to Dr. E. J. de Beer and Dr. George H. Hitchings for valuable discussions of this work.

SUMMARY

1. The comparative rates of oxidation by amine oxidase in cat and guinea pig liver Brei of a series of primary, secondary and tertiary phenethylamines, unsubstituted or substituted in the 2, 3 and 4 positions with hydroxy and methoxy groups have been studied.

2. Tertiary amines were less rapidly oxidized than primary and secondary amines and quaternary salts were not oxidized. Secondary amines were usually oxidized at the same or greater rates than the primary amines.

3. 2-Hydroxyphenethylamines were oxidized less rapidly than the 3- and 4-position isomers.

4. 2-Methoxyphenethylamines were oxidized more rapidly than the 3- and 4-isomers. 2,3-Dimethoxyphenethylamines had the most rapid rates of the dimethoxy derivatives.

5. The β -hydroxy group reduced the ease of oxidation.

6. The relationship of ease of oxidation by amine oxidase and pharmacological activity of the compounds was discussed.

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TOXICITY OF STABILIZERS FOR CONCENTRATED HUMAN ALBUMIN SOLUTIONS¹

I. PRODUCTION OF CARDIAC ARRHYTHMIAS IN CATS BY SODIUM SALTS OF CAPRYLIC AND RELATED FATTY ACIDS

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The heat stability of concentrated solutions of human serum albumin can be significantly increased by the addition of a variety of nonpolar anions in low concentration (1). Knowledge of the toxicity and pharmacodynamics of these substances is inadequate for judging whether they can be safely administered intravenously in the treatment of hemorrhage, shock and related conditions in human beings. This series of papers will report investigations designed to extend such information relative to sodium caprylate, caproate, mandelate and acetyl-tryptophanate.

The present paper is concerned with the abnormalities of cardiac function produced by the sodium salts of the lower fatty acids.

METHODS. The experiments were performed on cats anesthetized with 37.5 mgm. per kg. of pentobarbital injected intraperitoneally. Tracheal cannulae were inserted. Arterial pressure was recorded from a carotid artery by means of a mercury manometer, sodium thiosulfate being used as an anticoagulant. A cannula was inserted into a femoral vein for intravenous injection. Electrocardiograms were obtained in many experiments by means of a Sanborn Cardiette.

RESULTS. A. MODERATE DOSES. The concentration of sodium caprylate recommended by Luck and associates for stabilization of 25% human serum albumin solutions is 0.025M. The contents of two 100 cc. ampules of such a solution—a typical quantity used in the treatment of hemorrhage or shock in man—corresponds approximately to 3 cc. per kg. Accordingly we adopted 3 cc. per kg. as the standard dose of caprylate-albumin solutions in our work. Following these injections the arterial pressure rose on the average 18 mm. Hg. The maximum was reached in 3 to 5 minutes, and the pressure returned to normal in about 20 minutes. Since the pressure rise was the same with 0.075M, 0.15M and 0.25M caprylate solutions, it is probably due to the fluid and protein injected, rather than the caprylate itself.

A dose of 3 cc. per kg. 0.025M sodium caprylate in 25% human albumin given to cats intravenously produced no effects other than those to be expected on the basis of the protein and fluid injected.

When the concentration was increased tenfold, i.e., to 0.25M, disturbances of

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

cardiac rhythm—multiple ventricular ectopic systoles, various types of coupled beats (typically pulsus bigeminus) and idioventricular rhythms—commonly appeared.

Incidence of caprylate arrhythmia in relation to dosage.—Each of fifteen cats was given four consecutive doses of 3 cc. per kg. of a fixed concentration of sodium caprylate at twenty-minute intervals. The incidence of arrhythmia following one or more of these doses is shown in the following table.

CONCENTRATION OF CAPRYLATE, M	NUMBER OF CATS	ARRHYTHMIA		PERCENTAGE OCCURRENCE
		Present	Absent	
0.075	4	0	4	0
0.15	4	1	3	25
0.25	7	5	2	71

From these data and the assumption that the incidence would be proportional to the logarithm of the dose, it has been estimated by the use of Bliss' probit method of biological assay (2) that in cats under the conditions of our experiment, arrhythmia might be produced by caprylate, in the concentration (0.025M) recommended for albumin stabilization, four times in a million.

Related fatty acids. Since the tendency of sodium caprylate, in the higher concentrations used, to produce cardiac arrhythmias might be considered as a contraindication to its clinical use as a stabilizer for albumin solutions, it was thought advisable to determine whether related fatty acids, which are also effective albumin stabilizers, might be relatively free of this disadvantage.

Accordingly sodium caproate ($\text{CH}_3(\text{CH}_2)_4\text{COONa}$), sodium heptylate ($\text{CH}_3(\text{CH}_2)_5\text{COONa}$) and sodium caprate ($\text{CH}_3(\text{CH}_2)_8\text{COONa}$) were given to cats in the same high dosage (3 cc. per kg. 0.25M in albumin) as that of sodium caprylate ($\text{CH}_3(\text{CH}_2)_6\text{COONa}$) with the results shown in the following table.

Incidence of cardiac arrhythmia produced by lower fatty acids

	NO OF C ATOMS	NO OF CATS	ARRHYTHMIA		PERCENTAGE OCCURRENCE
			Present	Absent	
Sodium caproate	6	4	1	3	25
Sodium heptylate	7	5	2	3	40
Sodium caprylate	8	7	5	2	71
Sodium caprate	10	4	4	0	100

From these results it appears that the tendency of the sodium salts of the lower fatty acids to produce cardiac arrhythmias increases with the number of carbon atoms in the fatty acid chain. This rule has been reported to describe other biological actions of these fatty acids: the induction of artificial parthenogenesis in the sea urchin egg (3), retraction of the posterior end of the earthworm (4), swelling with cessation of ciliary movement of the ciliate *Euplotes* (5), closure of the rock barnacle (6), and cessation of mouth movements in the sunfish (7).

It therefore appears to be of general physiological validity. Various hypotheses concerning the relation of the physicochemical properties of these acids, such as their capacity to reduce surface tension, their lipid solubility, etc., to their physiological effects are discussed by these authors.

Fatty acids also tend to produce hemolysis, this tendency increasing with length of the carbon chain (8, 9). The critical concentration at which caprylate just induces hemolysis *in vitro* is 0.1M. Since this concentration is much above that attained after mixing in the blood stream after an injection of caprylate (10), it is improbable that significant *in vivo* hemolysis occurred in our experiments.

DESCRIPTION OF THE ARRHYTHMIAS. Time relations. The time from the beginning of the injection to the onset of arrhythmia varied from 37 to 165 sec. (average, 100 sec.). The duration varied from 3 to 38 min. (average, 11 min.).

A peculiarity worthy of note is the fact that, as a general rule, the arrhythmia, if it occurred at all, did so following the first or second injection, but not following the third or fourth injection. We have no explanation for this finding.

Electrocardiographic analysis. The cardiac arrhythmias provoked by the lower fatty acids were not constant in type but varied between individual cats and at different times in the same cat. The principal types observed were:

- (1) irregularly occurring single ventricular extrasystoles in an otherwise normal sinoatrial rhythm (e.g., fig. 2, B);
- (2) regular interpolation of ventricular extrasystoles between one, two or three normal systoles (pulsus bigeminus (fig. 1, B), trigeminus (fig. 3, B) or quadrigeminus, or of more than one extrasystole following each normal systole (fig. 1, C and D));
- (3) idioventricular rhythms consisting of ventricular extrasystoles without systoles of supraventricular origin (fig. 2, D).

Of these pulsus bigeminus was by far the most common. Typically, a normal rhythm was suddenly replaced by pulsus bigeminus, which later spontaneously gave place to a normal rhythm with equal suddenness.

However, in certain experiments there was an orderly progression from type (1) through type (2) to type (3). Such a progression suggests that the fatty acid tends to establish ectopic ventricular foci and that, as the intensity of its action increases, these foci succeed in discharging impulses more and more frequently until they become the sole pacemaker for the ventricles.

A particularly interesting example of the waxing and waning of the tendency toward production of ectopic beats occurred in Expt. No. 25 (fig. 1) in which sodium heptylate was given. The first arrhythmia to appear was a typical pulsus bigeminus (fig. 1, B), which was followed by groups of beats in which each normal systole was succeeded by two, three (fig. 1, C) or even six (fig. 1, D) ectopic systoles, which, judging from their varying contours, arose from at least three different ventricular foci. Shortly afterward the complex coupled rhythm gave way to simple pulsus bigeminus from a single ectopic focus (fig. 1, E), then to a sinoatrial rhythm with irregularly interspersed extrasystoles, and finally to an entirely normal rhythm (fig. 1, F).

In this case there was, over the period of twenty or thirty systoles at least, a remarkable degree of stability of functioning among the foci originating ectopic beats. As shown in figure 1, D each normal beat was followed by six ectopic

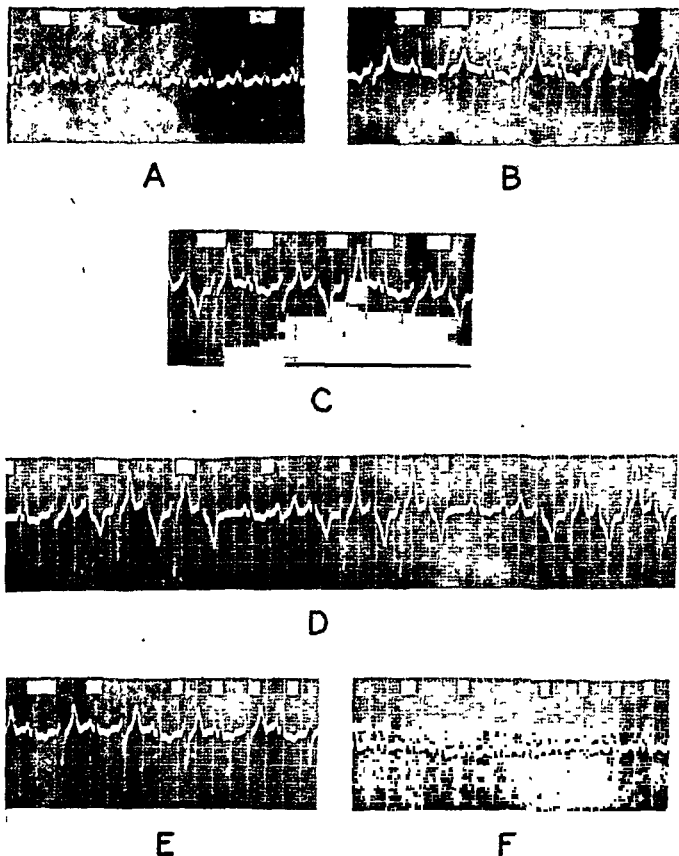


FIG 1. EKG CHANGES FOLLOWING SODIUM HEPTYLATE INJECTION

Expt. No. 25, Lead II. Sequence following a single injection. A. Normal before injection. B. Pulsus bigeminus. C. Supraventricular beat followed by three extrasystoles from more than one focus. D. Supraventricular beat followed by six extrasystoles from several foci. E. Pulsus bigeminus. F. Normal rhythm.

systoles of at least three different contours (presumably therefore from at least three different foci), this pattern being repeated identically three times in the record.

A further characteristic of the ectopic ventricular beats, noted in several

experiments, was the change in contour, as the arrhythmia proceeded, from the wide diphasic form to one approximating the QRS complex of the supraventricular beats. This change suggests that the ectopic pacemaker shifts from a posi-

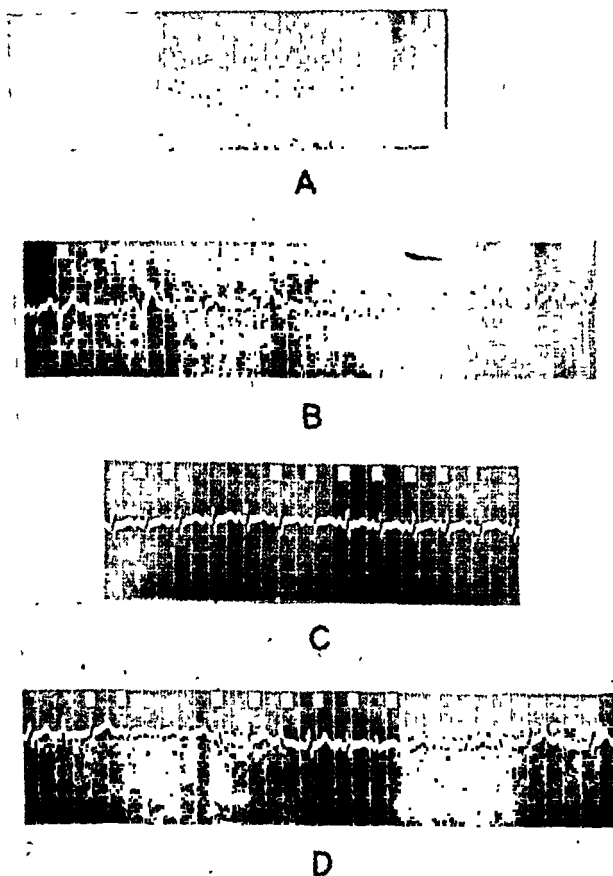


FIG. 2. EKG CHANGES FOLLOWING SODIUM CAPRYLATE INJECTION

Expt. No. 31. Lead II. Sequence following single injection. A. Normal before injection. B. Supraventricular beats with two types of extrasystoles. C. normal rhythm (after injection) with beats from two ectopic foci at a gross rate of 210 syst/min. Complete atrioventricular dissociation. D. Complete atrioventricular dissociation at rate of 196 per min.

tion in the ventricular mass to one in the atrioventricular conducting system. An example of this change is seen in figure 3, B and D.

The normal QRS complexes undergo no gross change from their preinjection form under the influence of the fatty acids, either when no arrhythmia appears

or when the normal complexes occur among ectopic ventricular beats. There may, however, be occasionally an inversion of the T wave.

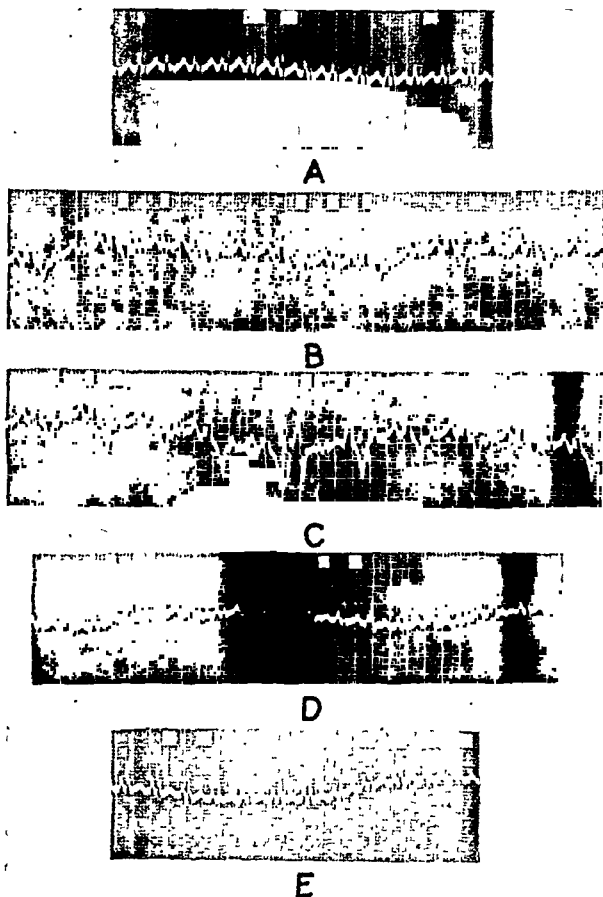


FIG. 3. EKG CHANGES FOLLOWING SODIUM CAPRYLATE INJECTION

Expt. No. 35. Sequence following single injection. A. Normal before injection (Lead II). B. Pulsus trigeminus (Lead II). C. Pulsus bigeminus (Lead III). D. Pulsus bigeminus of origin apparently close to atrioventricular node (Lead II). E. Subsequent normal rhythm (Lead II).

It may be concluded that the sodium salts of the lower fatty acids tend to set up foci in the ventricular muscle (or in the atrioventricular conducting system) which at first give rise to occasional isolated extrasystoles, then to extrasystoles coupled with normal beats, and finally to extrasystoles at a rate high enough that the focus (or foci) becomes the dominant pacemaker of the ventricles.

Pulse rate and arterial pressure changes accompanying the arrhythmia. When the arrhythmia takes the form of pulsus bigeminus, the pulse rate (P), obtained from the kymographic record of arterial pressure, drops to a value exactly one half that of the ventricles (V), obtained from the simultaneous electrocardiographic record, the extrasystolic beats apparently failing to open the aortic valves. With pulsus trigeminus a 3:1 V-P rhythm may occur. With irregular rhythms an even greater pulse deficit may appear. Finally, during an idioventricular rhythm a 2:1 V-P ratio may occur without irregularity in the intervals between the systoles.

The onset of such arrhythmias is accompanied by a sudden fall in the mean arterial pressure level of about 10 mm. Hg, which endures as long as the arrhythmia persists.

Obvious factors responsible for the pulse deficit include: (1) the prematurity of the extrasystolic beats reduces the filling time and accordingly the strength of the beat; and (2) asynchrony in the contraction of the different parts of the ventricles excited by the abnormal spread of excitation from the ectopic focus with consequent subnormal peak intraventricular pressure development.

Conditions favoring the development of the arrhythmia. Neither the absolute level of arterial pressure before injection nor the rise in pressure occurring on injection appeared related to the occurrence of arrhythmia. In the presence of a high heart rate, however, the arrhythmia is likely to develop: of eight cats given caprylate, four showed arrhythmia; their heart rates averaged 288 per min. (range, 192-456); the heart rates of those failing to show arrhythmia averaged 168 per min. (range, 114-240). Again, when the arrhythmia followed either the first or the second caprylate injection, but not both, it always occurred after the injection accompanied by the faster heart rate. These findings suggest the possibility that, when the cardiac sympathetic is active, arrhythmia may be more readily provoked. If this should be the case, the arrhythmia produced by fatty acid salts would be comparable with those produced by a variety of procedures—inhalation of chloroform (11); chemical, mechanical or electrical stimulation of the nasal mucosa (12); electrical stimulation of the hypothalamus (11); and intracisternal injection of potassium salts (13)—all of which are ineffective after interruption of the sympathetic pathway between the brain stem and heart.

Arrest of pulsus bigeminus by potassium chloride injection. In three cats showing pulsus bigeminus as the result of fatty acid administration, the injection of 1 cc. of 0.15M potassium chloride intravenously promptly abolished the ectopic ventricular beats. In one case, the arrhythmia was demonstrated five times, the pulsus bigeminus returning spontaneously in 1 to 3½ min. after each potassium injection. In one of these tests a continuous EKG record was obtained. It showed that pulsus bigeminus disappeared 3.48 sec. from the beginning of the potassium injection. The only transitional change found was that the last ectopic beat had a somewhat reduced R voltage.

We have estimated from data in the literature (14) that the dose of potassium chloride employed could hardly have raised the blood potassium content by more than 1.2 mM per l., a rise too small to evoke demonstrable EKG changes in

their experiments. However, the effectiveness of potassium as a cardiac depressant is related rather to the rate of rise of serum potassium concentration than to its absolute level (15). Since the contour of the ventricular beats of sinus origin was not altered, it is improbable that the potassium abolished the ectopic beats by evoking intraventricular block. Rather, the action would seem to have been a direct depression of the automatic rhythmicity of the ectopic focus. Such a depressant effect of potassium on the rhythmicity of parts of the heart other than the normal pacemaker has been demonstrated (16).

Failure of hemorrhage to increase tendency to caprylate arrhythmia. Since the fatty acids (especially caprylate) were under consideration as stabilizers for albumin solutions to be given to men with hemorrhage or shock, it was considered advisable to determine whether caprylate might have a greater tendency to produce cardiac arrhythmias in the presence of such disorder than in normal animals.

Accordingly, cats, prepared as described above, were subjected to a rapid hemorrhage from the femoral artery which reduced their arterial pressures to about 65 mm. Hg. At five-minute intervals, subsequent hemorrhages were induced to bring the pressure back to this level. After about one half hour the pressure finally showed little tendency to rise above this level. A dose of 3 cc. per kg. of 0.15M sodium caprylate in albumin was then given. After twenty minutes, the pressure was again reduced to the 65 mm. Hg level by bleeding and another dose of caprylate in albumin given. This was repeated until four doses had been given or the cat had died.

Four such experiments have been performed. In no case did arrhythmia develop. Since 0.15M sodium caprylate produced arrhythmia in one of four normal cats and in none of four cats subjected to bleeding, it seems probable that hemorrhage does not increase the susceptibility to caprylate-induced arrhythmia.

B. LARGE DOSES. Doses of 7.2 cc. per kg. of 0.25M sodium caprylate (40 times the dose recommended for clinical use) given intravenously caused death within a few minutes in all three cats tested.

Within half a minute of the beginning of the injection the arterial pressure began a fall which continued uninterruptedly to death of the animal. The respiration stopped permanently when the arterial pressure had declined about 5 mm. Hg. The heart rate now began to slow. This was associated with prolongation of the P-R interval, indicative of early depression of atrioventricular conduction. Sometimes, but not invariably, inversion of the T wave occurred at this time. Otherwise the EKG showed nothing unusual at this stage.

When the arterial pressure had reached about 55 mm. Hg, the EKG showed in rapid succession 2:1 A-V block and then complete A-V block with idioventricular rhythm. In several cats the amplitude of the T wave rose very markedly for a brief period. The P waves slowed and then disappeared. The ventricular complexes often accelerated but, as the arterial pressure fall continued, they suffered decline in amplitude and became wider. At this time the amplitude of the T wave exceeded that of the R wave in certain instances. Finally complete cardiac arrest occurred at about five minutes from the time of injection.

One cat, anesthetized with ether and given 6.3 cc. per kg. of 0.25M sodium caprylate (35 times the recommended dosage) underwent immediate cardiovascular collapse with cessation of respiration and heart action. Some seconds later, however, it began to make forceful inspiratory gasps at a rate of 4 to 5 per min., and the heart sounds became weakly audible. Respiration and heart action improved gradually and in about five minutes were apparently normal. The cat recovered from the ether anesthesia promptly and showed no abnormalities on the next day or thereafter. Apparently, if the animal survives the acute collapse, it will show no subsequent obvious ill effects.

Sodium caproate produced effects qualitatively similar to those of sodium caprylate. In general, however, cats survive considerably higher doses of the former salt. For example, one cat given 54 cc. per kg. of 0.25M sodium caproate showed no immediate or delayed ill effects. This is in line with the smaller incidence of arrhythmia following low doses of caproate than of caprylate.

The mechanism whereby the fatty acid salts cause the fall in arterial pressure and cessation of respiration has not been determined. The slowing of A-V conduction, followed by the development of partial, then complete A-V block observed to accompany the arrest of breathing and the decline in arterial pressure (and consequent impaired coronary flow), are very similar to those occurring in the collapse stage of anoxic anoxia in intact animals (7). Myocardial anoxia obviously results from both conditions. This, rather than any specific action of the fatty acid salts, is probably responsible for these later disturbances.

Earlier characteristic electrocardiographic signs of anoxic anoxia in intact animals are a decrease in the T wave amplitude and a depression of the S-T segment. These have not been observed in the early stage of vascular collapse after fatty acid administration. However, these changes are presumably the result of the hypocapnia evoked by the anoxia rather than of the myocardial anoxia *per se* (18). Since the fatty acid salts, injected at a pH of 7.3, would not evoke hypocapnia, it is not surprising that the T wave and S-T segment changes did not appear in these experiments.

Discussion. *Mechanism of production of ventricular ectopic rhythms by the lower fatty acids.* Several of the current hypotheses seeking to explain the genesis of ventricular ectopic rhythms depend on the postulation of various specific forms of intraventricular block. Since the width and contour of the QRS complexes of the supraventricular beats is not altered in the presence of the arrhythmia we have studied, intraventricular block may be excluded. (Atrioventricular block, however, is sometimes evoked.)

Other hypotheses postulate an increase in the automaticity of a ventricular focus (or foci) to the point that it becomes the ventricles' pacemaker. That this can occur following fatty acid administration is clearly shown by the finding, in Expt. No. 34, of a regular idioventricular rhythm from the ectopic ventricular pacemaker with a rate higher than the initial heart rate and higher than that of the atria, total atrioventricular dissociation being present (fig. 2, D).

The process underlying the increase of automaticity of the ventricular foci is not known. Application of sodium salts of caproic, caprylic and capric acid causes depolarization of frog muscle and frog and crab nerves, as shown by the

development of a negative membrane potential, the effectiveness increasing with the length of the fatty acid chain (19). This effect might conceivably render the rhythmic "prepotentials" (20) of cardiac muscle supraliminal and so create a rhythmically discharging focus.

Examples of excitation of a number of lower organisms by these substances have already been cited. To these may be added the production of convulsions by the application of caprate and valerate directly to the spinal cord of the frog (21).

Similarity of the arrhythmia to that produced by digitalis. It is well known that digitalis in toxic doses can evoke ectopic rhythms (multiple isolated ventricular extrasystoles, bigeminal or other coupled rhythms and ventricular tachycardias), as well as various forms of A-V block, disturbances also produced by administration of the fatty acid salts. Moreover, small doses of potassium salts abolish the arrhythmias produced by digitalis (22), as they do those produced by the lower fatty acids.

However, the changes in the S-T segment and T wave characteristic of digitalization are not produced consistently by these substances.

Clinical use of fatty acids as stabilizers of human albumin solutions. The clinical occurrence of multiple ventricular ectopic beats, particularly in the form of coupled beats, such as pulsus bigeminus, is often regarded as of serious prognostic import, since it may be premonitory of paroxysmal ventricular tachycardia or even of ventricular fibrillation (23). The production of such arrhythmias by sodium caprylate or sodium caproate might appear to contra-indicate their use as stabilizers of albumin solutions. However, in our experience the arrhythmia has never lasted beyond a half hour—possibly because of rapid metabolic removal of the fatty acid from the blood stream—and in no case has it led to ventricular fibrillation.

Further, the dosage required for the evocation of arrhythmia is so much greater than that required for stabilization that the chances of its occurring, at least under the conditions of our experiments, are vanishingly small. Even when the enormous doses causing transient cardiovascular collapse are given, the animals show no apparent subsequent ill effects.

Finally, since the conditions for which intravenous injection of stabilized albumin solutions are more commonly indicated, hemorrhage and shock, are accompanied by a rise in plasma potassium concentration, and since, as we have shown, potassium arrests the arrhythmia caused by the fatty acid stabilizers, the chances of the arrhythmia occurring under actual clinical use are definitely less than when they are given to normal subjects.

SUMMARY

The sodium salts of the lower fatty acids (caproic, heptylic, caprylic and capric) in concentrations ten times those recommended for stabilization of concentrated human albumin solutions tend to evoke ectopic ventricular beats (isolated extrasystoles, coupled rhythms and ventricular tachycardias) when given intravenously to cats. The tendency increases with the length of the fatty acid chain.

Potassium salts abolish these arrhythmias as they do the similar ones caused by toxic doses of digitalis. Hemorrhage does not increase the incidence of these cardiac disturbances. The arrhythmias are attributed to an increase in the automatic rhythmicity of ventricular foci. Since the chances of occurrence of the arrhythmia under the conditions of clinical use are vanishingly small, this toxic action of the fatty acids salts is not considered to contraindicate their use in the concentrations which are effective as stabilizers of concentrated albumin solutions.

The authors wish to acknowledge the stimulating leadership of Dr. J. M. Luck, Responsible Investigator of the project, and of Dr. Paul Boyer, whose advice and assistance in the chemical aspects of the work was invaluable.

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PROTECTION OF CHOLINESTERASE AGAINST IRREVERSIBLE INACTIVATION BY DI-ISOPROPYL FLUOROPHOSPHATE IN VITRO

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Koster (1) has reported a seemingly paradoxical phenomenon in regard to the mutual effects on cats of di-isopropyl fluorophosphate (DFP), an irreversible anticholinesterase agent (2), and eserine, which inhibits cholinesterase reversibly (3). Following the injection of a single dose of DFP, a marked and lasting sensitivity to eserine was produced, but when a non-lethal dose of eserine was given first, it afforded protection against several otherwise lethal doses of DFP. The increased toxicity of eserine following the previous administration of DFP is to be expected from the fact that a large portion of the tissue cholinesterase would be inactivated at the time when eserine was given. As a result, only a small amount of cholinesterase would have to be inactivated by eserine to produce death (4). On the other hand, protection against one anticholinesterase agent by another is suggestive of the antagonistic effects which have been demonstrated between compounds similar in structure. Thus, Unna (5) has shown that N-allyl morphine offers protection against morphine, and the findings of Swan and White (6) indicate a competitive antagonism between acetylcholine derivatives and acetylcholine. It is conceivable that the protective action exerted by eserine when injected prior to DFP might result from the reversible combination of eserine with the active groups of the cholinesterase molecules, thereby blocking access to DFP and the subsequent formation of an irreversible complex. During the time necessary for the dissociation of the eserine-cholinesterase complex, part of the uncombined DFP would be excreted or hydrolyzed (7), and the liberated cholinesterase would then resume its physiological function. For the present report, this hypothesis has been tested by a series of *in vitro* studies on the protection afforded rat brain cholinesterase against DFP by eserine and nineteen other reversible anticholinesterase agents. The method employed, described below in detail, consisted in brief of adding DFP to incubated mixtures of the enzyme and inhibitor, incubating further, and dialyzing off the uncombined DFP and reversible inhibitor, after which the undialyzable residue was tested for cholinesterase activity.

METHODS. Following an initial experiment in which eserine was found to protect human serum esterase against DFP, all subsequent studies were done with rat brain homogenate prepared in a manner previously described (4). *In vitro* inhibition of cholinesterase by eserine and the other compounds investigated was determined in the Warburg apparatus. Mixtures of homogenate and various concentrations of eserine were incubated in bicarbonate buffer in the main wells of the vessels at 38°C. for twenty minutes, after which acetylcholine bromide solution was tipped in and CO₂ liberation followed. The details of this

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method have already been published (4). To determine the protection against DFP produced by eserine, 1.6 cc. of homogenate (1 part wet weight of tissue in 6) were incubated with 0.2 cc. of the appropriate concentration of eserine to produce the desired final concentration for twenty minutes, after which 0.2 cc. of 10^{-3} M DFP was added. (This final concentration of DFP normally produces 95 to 98 per cent inhibition of rat brain cholinesterase.) Following an additional incubation period of thirty minutes, the mixtures were transferred quantitatively to collodion tubes and allowed to dialyze against running tap water at 12-13°C. for sixty-four hours. The contents of each dialysis tube were then transferred to a graduated test tube, 1.0 cc. of 0.4 M $MgCl_2$ added to replace the removed cations, and the volume was brought up to 10.0 cc. with distilled water. Cholinesterase determinations were run on 2.0 cc. aliquots. The other compounds were studied in the same manner, except that they were allowed to incubate for thirty minutes with the homogenate before the addition of acetylcholine bromide or DFP in the inhibition or protection experiments respectively. Variations from the above figures for the volumes of the homogenate and reversible inhibitor solutions were introduced when high concentrations of the latter were employed. In the cholinesterase determinations, corrections were made for hydrolysis of the inhibitors themselves and non-enzymatic hydrolysis of acetylcholine. Additional dialyses were always run on mixtures of homogenate and DFP, homogenate and reversible inhibitor, and on homogenate alone. The activity of the last, which was found to be reduced to approximately eighty per cent of its original value by dialysis, was used as the control for calculating the activities of the other dialyzed preparations. The portions of homogenate incubated with DFP alone showed from one to five per cent activity after dialysis. As indicated in table 1, the inactivation produced by some of the inhibitors, which are reversible at relatively low concentrations, could not be reversed by dialysis at the highest concentrations employed, and in many instances probably represented denaturation of the protein enzyme. In all other cases, at least ninety per cent of the control activity was restored.

RESULTS AND DISCUSSION. As Roepke (8) and Straus and Goldstein (9) have noted previously, the addition of acetylcholine to the cholinesterase-eserine complex resulted in the gradual displacement of the inhibitor by the substrate. Consequently, the initial velocities of acetylcholine hydrolysis following incubation with eserine were much lower than values subsequently attained, particularly in the presence of the lower concentrations of eserine. In the inhibition curve (I) in figure 1, the values given for cholinesterase inhibition are those obtained during the ten minute interval beginning twenty minutes after the addition of acetylcholine to the cholinesterase-eserine complex. Two criticisms of this curve are apparent. Twenty minutes incubation is not sufficient to permit the attainment of equilibrium between eserine and cholinesterase (9), but it was desired to reproduce as nearly as possible the conditions employed in the *in vivo* studies of Koster (1), where a similar or shorter interval between eserine and DFP administration was found to afford maximal protection. Likewise, equilibrium between cholinesterase, eserine and acetylcholine was not established after thirty minutes reaction; however, this interval was selected as it represented the end of the incubation period employed in the protection experiments where the attempt was made to simulate time conditions in the body.

From the protection curve (II) in figure 1 it may be seen that eserine protects cholinesterase against irreversible inactivation by DFP. The degree of protection against a constant concentration of DFP varied with the concentration of eserine used, and at the highest concentration tested (10^{-2} M), protection was complete. The fact that the two curves, which are roughly parallel, do not

coincide indicates that during the incubation period after the addition of DFP, the latter compound was more effective than acetylcholine in displacing eserine from the cholinesterase. However, this result is probably partially due to the fact that reaction between the enzyme and DFP continued at a reduced rate at the lower temperature of the dialysis bath until the free DFP was completely hydrolyzed or dialyzed off.

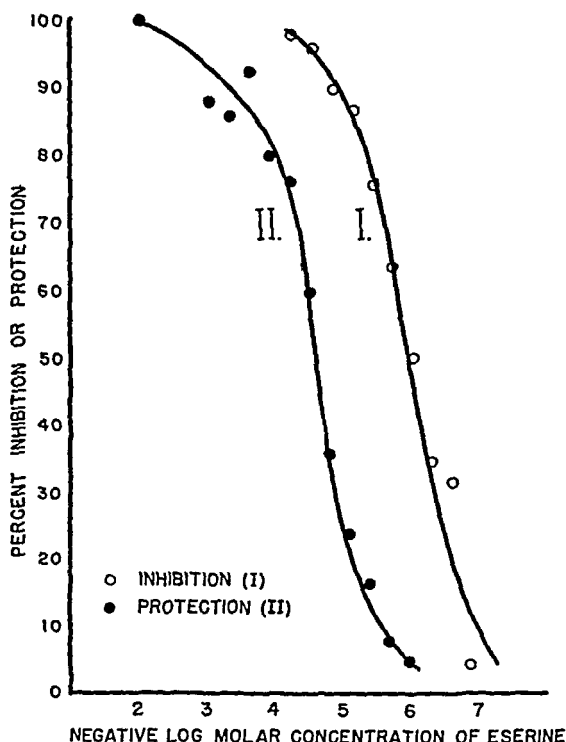


FIG. 1. INHIBITION OF RAT BRAIN CHOLINESTERASE BY ESERINE AND PROTECTION AFFORDED AGAINST 10^{-4} MOLAR DFP

In a previous study (4), it was found that the brain cholinesterase of rats could be reduced to below twenty per cent of normal by the administration of DFP without producing noticeable symptoms. It will be seen in figure 1 that a concentration of eserine (10^{-5} M) sufficient to inhibit eighty-five per cent of the cholinesterase (curve I) protected approximately twenty-five per cent of the total amount against irreversible inactivation by DFP (curve II). Inasmuch as both eserine (10) and DFP (7) are rapidly destroyed in the body, the displacement of the reversible inhibitor by DFP would not be expected to proceed to such an extent *in vivo* as it did in the present experiments. Thus, in the organ-

TABLE 1

Protection of rat brain cholinesterase against DFP by reversible anticholinesterase drugs in vitro

COMPOUND	NEGATIVE LOGARITHM OF MOLAR CONCENTRATION	RAT BRAIN CHOLINESTERASE	
		Per cent inhibition	Per cent activity restored after incubation with DFP and dialysis
DFP control	4.0	95-98	5-2
Prostigmin bromide	4.0	98	96
Eserine salicylate	4.2	98	76.5
	5.1	87	24
Carbamylcholine chloride	1.0	100	88
	2.0	88	52
	3.0	26	0
Pilocarpine nitrate	1.0	100	20
	2.0	78	
Nicotine	1.0	98	15
	2.0	80	9
Atropine sulfate	1.0	58	12
	2.0	2	
Choline chloride	1.0	70.5	12
	2.0	24	
Morphine sulfate	1.0	100	9
	2.0	99	0
Procaine hydrochloride	1.0	100	12
	2.0	43	
Methylene blue	3.0	99	*
	4.0	78	<5
Strychnine sulfate	2.0	100	<5
	3.0	73	<5
Atabrine dihydrochloride	1.0	100	<5
	2.0	95	<5
	3.0	60	<5
Quinine dihydrochloride	1.0	100	*
	2.0	76	<5
Sodium fluoride	1.0	90	<5
	2.0	58	
Thiamin chloride	1.0	100	*
	2.0	29	<5
Cysteine	1.0	100	*
	2.0	12	<5
Sodium p-amino benzoate	1.0	100	*
	2.0	0	<5
Acetyl-beta-methylcholine chloride	1.0	58	<5
Acetylcholine bromide	1.0	45	<5
Intocostrin (curare)	10 units/cc.	67	<5

* Inactivation of cholinesterase in enzyme-inhibitor control not reversed by dialysis.

ism, following the administration of a protective dose of eserine and an otherwise lethal dose of DFP, the enzyme activity would be reduced below the critical value only from the time of combination of DFP with the unprotected fraction

of tissue enzyme until the dissociation of a certain portion of the cholinesterase-eserine complex. If the velocity of dissociation of this complex is such that sufficient enzyme would be liberated to hydrolyze acetylcholine before a lethal amount of the latter could accumulate, this mechanism could explain the protective effect of eserine against DFP *in vivo*. Further studies on this phase are being undertaken at present.

The results obtained with the nineteen other compounds are presented in table 1. They may be summarized as follows: only two compounds, besides eserine, gave marked protection (prostigmin and carbamylcholine); six showed slight but appreciable protective activity (pilocarpine, nicotine, atropine, choline, procaine and morphine), and the remaining eleven showed none (methylene blue, strychnine, atabrine, quinine, sodium fluoride, thiamin, cysteine, sodium p-aminobenzoate, acetyl-beta-methylcholine, acetylcholine and Intocostin). Although eserine and prostigmin are the most potent anticholinesterases in the above group, it is readily apparent that this property alone does not account for the protection. Seven compounds which were reversible at concentrations sufficient to cause greater inactivation than carbamylcholine at $10^{-2}M$ (which afforded fifty-two per cent protection) afforded little or no protection (pilocarpine, nicotine, morphine, procaine, strychnine, atabrine, sodium fluoride). Of greater significance, probably, is the ability of the compounds in question to compete with DFP for the same active groupings of the cholinesterase molecule, and their relative affinities for such. Roepke (8) suggested that eserine, prostigmin and carbamylcholine might thus compete with the substrate (acetylcholine), and Goldstein (11) has shown since then that this is definitely the case with eserine. Mazur and Bodansky's (12) data indicate that such a competition occurs between acetylcholine and DFP, although once the latter has combined with the enzyme, it cannot be displaced. The failure of acetylcholine and acetyl-beta-methylcholine to protect the enzyme is presumably due to the fact that these compounds are rapidly hydrolyzed by it and consequently the complexes they form possess high dissociation constants.

The presence of a carbamate group in the three most active protectors (prostigmin, eserine and carbamylcholine) suggests that combination of the inhibitor with the enzyme may occur at this portion of the molecule. The significance of this grouping in anticholinesterase drugs has been emphasized previously (13). The results obtained here indicate that some portion of the DFP molecule may react with the same moiety of the cholinesterase molecule as the carbamate group of the reversible inhibitors.

SUMMARY

1. Eserine, by combining reversibly with rat brain cholinesterase, has been shown to protect the enzyme against irreversible inactivation by DFP *in vitro*. The degree of protection varied directly with the concentration of eserine used. It is concluded that this phenomenon might explain the mechanism by which prophylactic eserine protects cats against DFP poisoning *in vivo*.

2. Of nineteen other anticholinesterase drugs similarly tested, two showed

marked protection (prostigmin and carbamylcholine), six afforded relatively slight protection (pilocarpine, nicotine, atropine, choline, procaine and morphine) and eleven showed none (methylene blue, strychnine, atabrine, quinine, sodium fluoride, thiamin, cysteine, sodium p-amino-benzoate, acetyl-beta-methylcholine, acetylcholine and Intocostin). The protective property does not appear to depend on the potency of anticholinesterase activity alone, but also on the ability of a compound to compete with DFP for a specific active group of the cholinesterase molecule.

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THE EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON NORMAL HUMAN EYES¹

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The local application of di-isopropyl fluorophosphate does not cause permanent damage to the eyes of rabbits (1). Consequently studies on the extent of the ocular reactions in human beings were undertaken along with an attempt to evaluate the counter effect of homatropine and atropine.

MATERIALS AND METHODS. White males between the ages of 18 and 26 years volunteered for the tests. They had varying amounts of college education and were intelligent observers. Negroes were not used for these tests because of the relatively feeble effect of midriatics in the colored race.

The men were examined prior to exposure and the following data recorded: a) distant vision without correction measured with the Snellen chart, b) retinoscopy and static refraction under homatropine cycloplegia, and change in distant vision with correction, c) size of pupil, measured directly with a hand rule, d) near point of accommodation, determined with a Prince Rule, e) speed of accommodation taking the time in seconds required to read Jaeger 1 at 18 inches after looking at infinity for several seconds. The six men in Group 2 were taken to a rifle range one week prior to their exposure where they fired for record on a target at 200 yards.

A static gas chamber was used. Group 1, composed of eight men were exposed to an analytical concentration of 19 micrograms per liter for 8½ minutes (CT 165) of di-isopropyl fluorophosphate. One of these men wore tightly fitting goggles. One man was not studied in detail but did give a verbal report of his symptoms. Group 2, consisting of 6 men were exposed 1 week later to an analytical concentration of 27.1 micrograms per liter for 9 minutes (CT 243.9).

Immediately on exit from the chamber, the men were partially examined and at 45 minutes a complete re-examination of their eyes was made.

This examination was repeated in Group 1 between 3 and 4 hours after exposure. Group 2 was taken to the rifle range where, after 4 hours, they fired for record. Both groups were re-examined at 1, 2, 3, 4, and 7 days after the test.

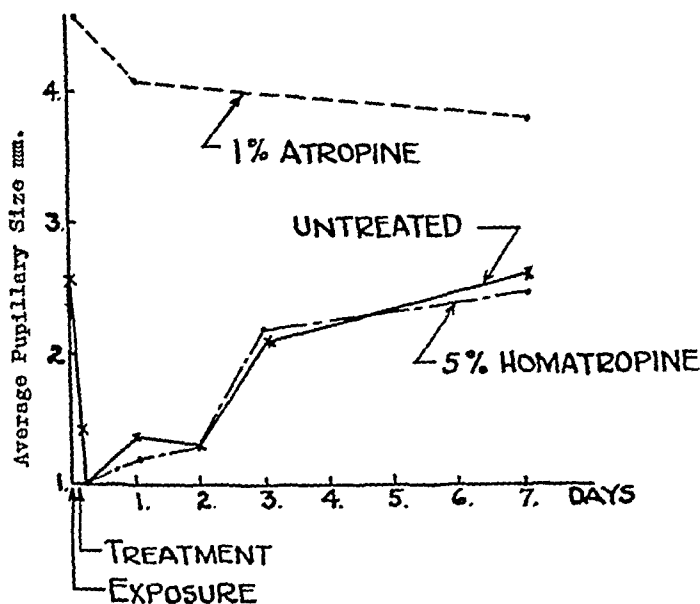
After the 45 minute examination, 3 men of Group 1 had 1% atropine sulfate instilled in their right eyes and 3 men received 5% homatropine hydrochloride. Since this amount of atropine paralyzed the accommodation and since the homatropine had no effect, Group 2 were given one drop of 0.25% atropine sulfate in the left eye on the first day after exposure.

RESULTS. Distant vision and refraction. The one volunteer whose eyes were protected with goggles maintained normal vision and had no ocular signs or symptoms throughout the period of observation. Prior to exposure all of the men in Group 1 had normal vision without correction, while 2 men in Group 2 required myopic corrective lenses to produce normal vision. The majority of

¹ The work described in this paper was done in part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University.

the men who were exposed to the drug showed diminished acuity of distant vision, and spasm of accommodation (false myopia of 1 to 6 diopters) which came on within 3 hours of exposure, and lasted 2 to 7 days, decreasing as the effect of the drug wore off. The duration of the visual disturbance was, on the average, greater in Group 2 than in Group 1.

Pupillary size and reaction. Immediately on exit from the chamber, all the men had small pupils (between 1 and 2 mm.) which failed to contract further on stimulation with a strong light. Measurements of the pupils of the men in Group 1 (graph 1) showed that the pupils began to relax between 1 and 3 days and attained normal size and activity between 3 and 9 days. The pupils of the

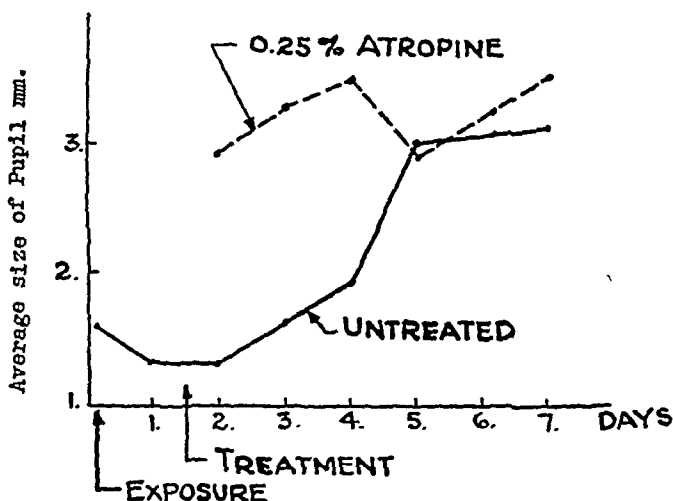


GRAPH 1. GROUP 1 (CT 160). EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON PUPILLARY SIZE AND RESULTS OF TREATMENT WITH 5% HOMATROPINE AND 1% ATROPINE

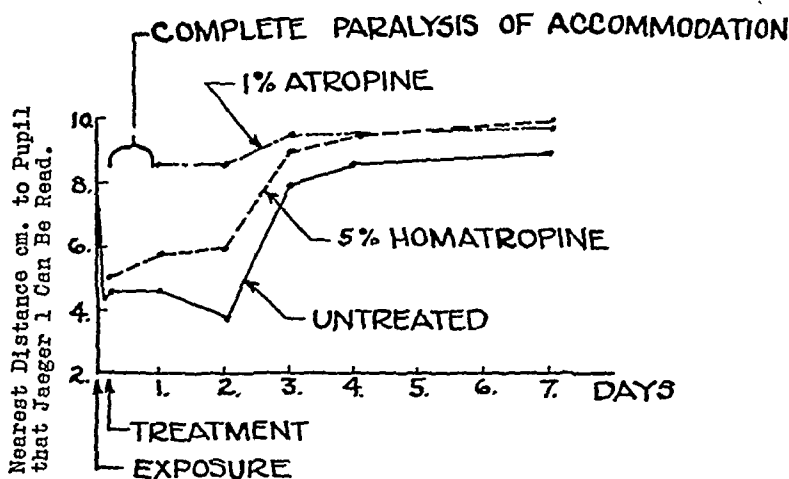
men of Group 2 (graph 2) did not begin to relax until after the third day and were not fully recovered until after 5 to 11 days. Less effect was noted on the pupils of the myopes in this group.

As the pupils of both groups began to return to normal size, it was noted that they were slightly oval with the long axis vertical and that they reacted sluggishly.

Conjunctival reaction. All of the men in both groups showed moderate diffuse conjunctival congestion which was not particularly confined to the intrapalpebral fissure. This gradually disappeared over a five day period in all of the men.



GRAPH 2. GROUP 2 (CT 244). EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON PUPILLARY SIZE AND RESULT OF TREATMENT WITH 0.25% ATROPINE SULFATE



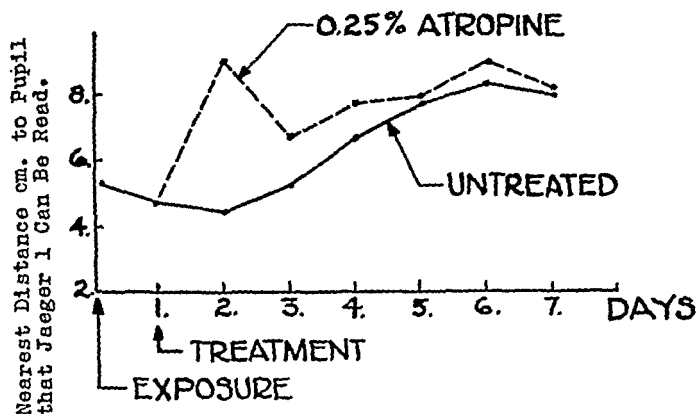
GRAPH 3. GROUP 1 (CT 160). EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON ACCOMMODATION AND RESULT OF TREATMENT WITH 5% HOMATROPINE AND 1% ATROPINE

Accommodation (graphs 3 and 4). The near point of accommodation was normal in all men in Group 1 prior to exposure. As the pupils constricted, the near point was moved closer so that within 5 to 15 minutes it was 3-6 cm. from the

cornea. Recovery of the untreated eyes gradually took place over an average of 3.5 days in Group 1, and 4.5 days in Group 2.

Concurrent with the shortening of the near distance, it became increasingly difficult for the men to focus after gazing into infinity. While Jaeger 1 print could be read when held at approximately 10 inches, several seconds were required before it could be seen clearly. Recovery from this slow accommodation took place simultaneously with the return to normal of the near point. Without exception these men complained of intense pain when they tried to perform visual tasks within 18 inches. Recovery from these signs and symptoms took place slightly earlier in those men exposed to a smaller CT.

Intraocular tension (graphs 5 and 6). In Group 1 the untreated eyes in all cases except one gave normal tension measurements at 5 minutes, slightly below normal at 3 hours and definitely lowered tension at 1 day. The one exception



GRAPH 4. GROUP 2 (CT 244). EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON ACCOMMODATION AND RESULT OF TREATMENT WITH 0.25% ATROPINE

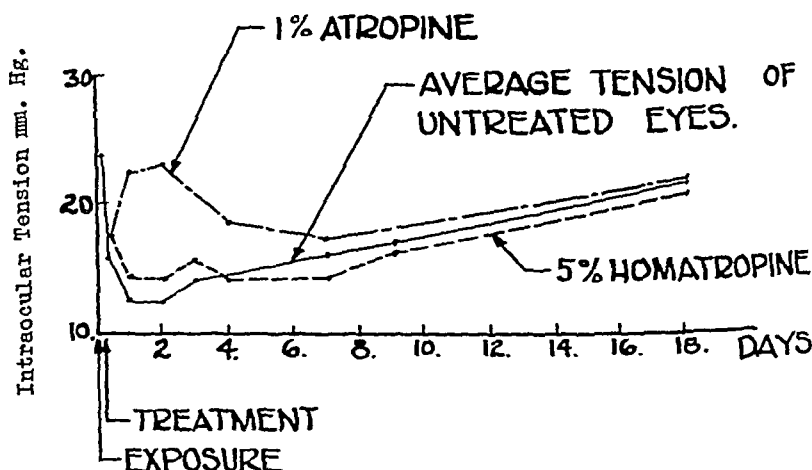
(R. L.) showed a slight rise in tension when measured at 10 minutes, but at 3 hours began to fall below normal. All of the eyes began to recover at 3 days and had almost normal tension at 9 days. At 18 days only three men were available for measurement, and these men had normal tension.

The Group 2 men showed a slightly lowered tension when measured 20 minutes after the exposure and markedly lowered tension for 3 to 4 days after which they began to slowly recover.

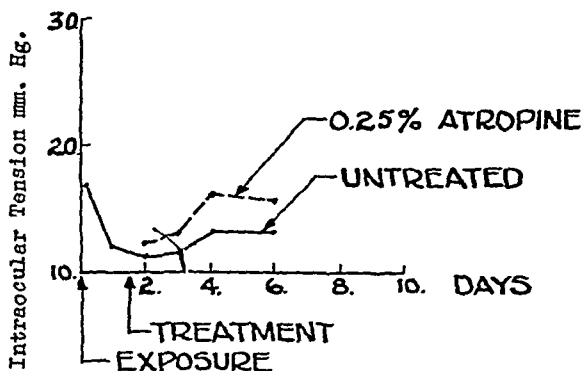
In summary, only one man (R. L.) out of the twelve had a transient rise in tension, the remainder showing a markedly lower tension which began to recover after the third day. The response of the men receiving the large CT was slightly longer than those exposed to the lower CT.

Performance test. Table 1 gives the results achieved by the 6 men of Group 2 when they fired for score on the 200 yard range before and after exposure to the

drug, and previous to the instituting of treatment. The difference in score was not significant although most of the men stated that they could not see the target distinctly.



GRAPH 5. GROUP 1 (CT 160). EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON INTRAOCULAR TENSION AND RESULTS OF TREATMENT WITH 5% HOMATROPINE AND 1% ATROPINE



GRAPH 6. GROUP 2 (CT 244). EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON INTRAOCULAR TENSION AND RESULTS OF TREATMENT WITH 0.25% ATROPINE SULFATE

General symptomatology. In Group 1 all of the men experienced a slight tightness of the chest, particularly noticeable in the first evening when attempting to smoke. Three of the men had no other symptoms and three had loose stools.

Four other men in the same barracks who had not been exposed to the drug also had mild gastrointestinal upsets at the same time. One of the exposed men (T. M.) had a history of asthma, nervousness and frequent attacks of fainting. On the first evening after exposure, he experienced some increased tightness in his chest and was one of the men above reported to have loose stools. By morning the tightness had increased and he experienced mild nausea. He was admitted to the hospital as a precautionary measure, the few asthmatic signs cleared in a few hours and he felt completely recovered. One of the men, in addition to having visual symptoms, experienced violent stomach cramps and frank diarrhea on the first night.

The reactions of the men in Group 2 were the same with the exception that no gastrointestinal signs were observed.

Effect of Treatment. a) Homatropine 5%. The men in Group 1 who received one drop of 5% homatropine after the 45 minute examination showed no significant difference between the treated and untreated eyes in respect to distant

TABLE 1
Results of group 2 men firing for score on the 200 yard range

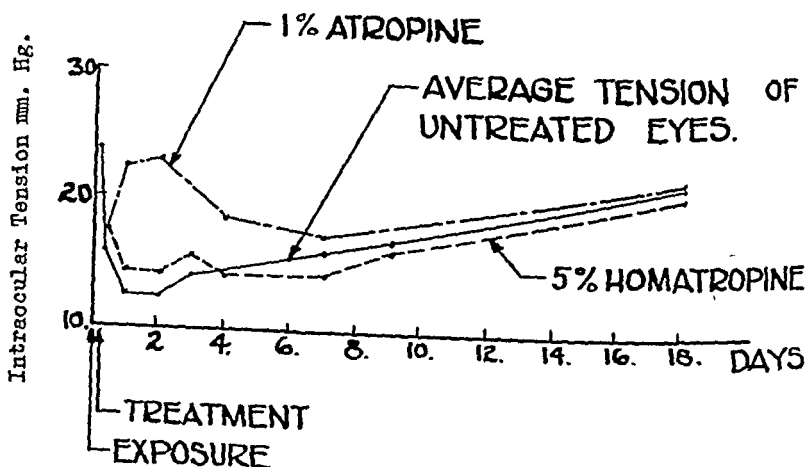
SUBJECT	2 WEEKS BEFORE EXPOSURE	4 HOURS AFTER EXPOSURE TO DI-ISOPROPYL FLUOROPHOSPHATE
J. P. L.....	150	144
J. P. D.....	146	154
J. J. H.....	146	151
H. S. H.....	166	147
R. A. K.....	138	143
A. C. D.....	123	110

vision, spasm of accommodation (graph 3), pupillary reactions (graph 1) or intraocular tension (graph 5).

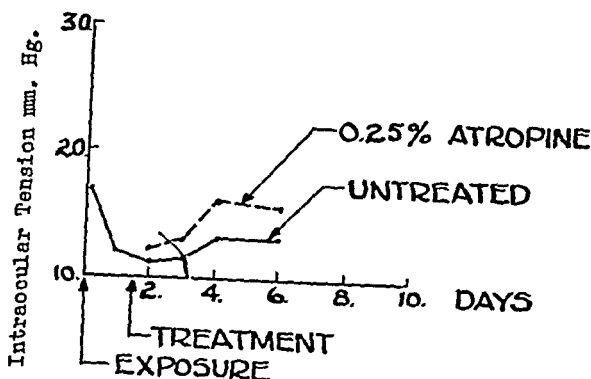
b) Atropine 1%. The eyes in the same group of men which received 1% atropine recovered from the false myopia in 3 hours showing normal distant vision without correction at this time. The control eyes in this group took 3.6 days to return to normal. One of the treated eyes showed a spasm of accommodation 1 day after exposure. On the other hand, this amount of atropine was sufficient to paralyze the accommodation (graph 3) for 24 hours after which accommodation returned to normal. The pupils of this group were dilated (graph 1) and remained so for 2 days and then returned to normal. The intraocular tension became normal at 24 hours and remained so for another 24 hours after which it again fell and then followed the same curve of recovery (graph 5) as the untreated eyes.

c) Atropine 0.25%. The left eyes of the 6 men in Group 2 were treated with 0.25% atropine sulfate 1 day after exposure. The hyperopic eyes in this treated group recovered from this false myopia and had normal distant vision in an average of 2.5 days while the untreated recovered in 5.3 days. There was no

drug, and previous to the instituting of treatment. The difference in score was not significant although most of the men stated that they could not see the target distinctly.



GRAPH 5. GROUP 1 (CT 160). EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON INTRAOCULAR TENSION AND RESULTS OF TREATMENT WITH 5% HOMATROPINE AND 1% ATROPINE



GRAPH 6. GROUP 2 (CT 244). EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON INTRAOCULAR TENSION AND RESULTS OF TREATMENT WITH 0.25% ATROPINE SULFATE

General symptomatology. In Group 1 all of the men experienced a slight tightness of the chest, particularly noticeable in the first evening when attempting to smoke. Three of the men had no other symptoms and three had loose stools.

5. A performance test showed no decrease in efficiency in markmanship.
6. The ocular signs and symptoms of the men exposed to a CT of 244 were almost completely relieved by a single application of 0.25% atropine sulfate.

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significant difference in the rate of recovery between the untreated and the treated myopic eyes. The near point of accommodation of these treated eyes was normal 24 hours after treatment and accommodation was not paralyzed as in those eyes in Group 1 which received 1% atropine sulfate (graph 4). Their pupils were of normal size within 24 hours (graph 2) and their tension returned to normal in 48 hours after treatment (graph 6).

The 5% homatropine only slightly relieved the intense pain experienced by those men whenever they focussed for near vision while the atropine in either 1% or 0.25% solution gave complete and prompt relief in the treated eyes in 3 hours.

DISCUSSION. The ocular effects of di-isopropyl fluorophosphate are produced by direct absorption of the agent through the exposed ocular tissues as is evidenced by the complete absence of signs and symptoms in the one man who wore goggles during exposure.

The most disabling signs observed in these men were the intense pain produced when they focussed on close objects coupled with the spasm of accommodation which produces a false myopia. The spasm of accommodation and the small pupil also moved the near point close.

Animal experiments (1) have shown that in rabbits under nembutal anesthesia there is a transient increase in intraocular tension followed by a lowered tension after exposure to the drug. Only one man gave evidence of a slight transient rise of pressure on the first (10 minutes) examination. All of the subjects including this one showed diminished intraocular pressure which lasted for several days after exposure.

The ocular signs and symptoms which are reported in detail in this paper constituted the major reactions of all the men observed. One man had a 12 hour attack of severe intestinal symptoms.

SUMMARY

1. Two groups of men were exposed to di-isopropyl fluorophosphate vapor for a CT of 165 and 244 respectively. Ocular signs and symptoms caused by direct conjunctival absorption were their main reactions to the exposure. The severity of the signs and symptoms varied slightly with the CT.

2. Nine of the twelve men studied had a diminution of distant vision caused by a spasm of accommodation. They recovered normal distant vision in from 3 to 7 days.

3. Effort at accommodation caused pain. Adjustment of vision from far to near and from near to far was slower than normal. The nearest point of clear vision and the farthest point of clear vision were both closer than normal. The men spontaneously recovered from these signs and symptoms in from 3 to 7 days. All men could read with moderate effort Jaeger 1 print at all times if it was held sufficiently close to the eyes.

4. Except for one man who had a transient rise in intraocular tension, all displayed a subnormal tension for several days.

stance, although an occasional one occurs in the white matter. They are very numerous in the cerebral cortex (fig. 1A) and they decrease in frequency through the brain stem (fig. 2B) and spinal cord (fig. 2A). These hemorrhages are confined to the grey columns in the spinal cord and are entirely perivascular in position. In the cerebellum the hemorrhages are limited to the cortex. They are



FIG. 1 Multiple hemorrhages occur in the nervous system of dogs made anemic by treatment with choline and carbamyl-choline. These are confined to the grey matter. A—A section through the cerebral cortex showing one larger hemorrhage and multiple petechiae. The nerve cells in this area show acute neurone degeneration. B—A section through the cerebellum showing hemorrhage into the Purkinje cell zone and adjacent area. There is marked destruction of the Purkinje cells along the line of hemorrhage. H. & E. 100X.

concentrated along the Purkinje cell zone, although they extend also into the adjacent molecular and granular layers (fig. 1B). The nerve cells show marked degeneration and destruction in the areas of hemorrhage. In addition, there is a generalized state of acute neurone degeneration which is more marked in the cerebral cortex. This change is characterized by chromatolysis and pyknosis of

NERVOUS SYSTEM CHANGES PRODUCED IN DOGS BY CHOLINE AND CARBAMYL CHOLINE¹

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It has previously been shown by one of us (1) that the daily feeding of Choline hydrochloride to dogs caused the development of a hyperchromic anemia which responded by therapeutic remission to liver extract injections, Stomach U.S.P., or Atropine Sulfate. More recently (2) we have found that such anemic animals also respond to treatment with folic acid.

The present study was undertaken to see whether carbamyl choline would produce a marked anemia in dogs, and also to study pathologically the nervous systems of dogs after treatment with choline and carbamyl choline. Since the production of anemia in these dogs has already been reported in abstract form (3) this paper will concern itself only with the nervous system changes which were found.

PROCEDURE. One splenectomized and five normal dogs were used as our experimental group of animals. Blood studies were made during a control period and continued throughout the period of experimentation.

Three of the dogs received two doses of Choline Chloride (10 mgm. per kgm., gastrically) daily for three weeks; then two doses of choline and two doses of physostigmine salicylate (0.2 mgm./kgm. subcutaneously) daily, for about sixteen days; and finally two doses of choline and two doses of carbamyl-choline, or doryl (.01 mgm./kgm. subcutaneously) daily for three or more weeks. Two dogs were fed choline three times daily (every six hours), and one dog was given three subcutaneous injections of carbamyl choline (doryl) daily, for five weeks. The physostigmine was tried because we thought that choline might act by forming acetyl choline, which is indirectly protected by eserine.

Four of the dogs were sacrificed during the experimental procedures at which time they were anemic, and two were sacrificed after being allowed to recover from the effects of medication, i.e., one five weeks after cessation of carbamyl choline, and one eleven weeks after choline treatment. Portions of the thoracic spinal cords and spinal nerves were taken from all six dogs, and the brains were removed from three dogs under sodium pentobarbital anesthesia (30 mgm. per kgm.). Brains and spinal cords from five untreated (normal) dogs were similarly removed. All tissues were immediately fixed in 10 per cent formalin solution.

Tissue from various portions of the cerebral cortex, diencephalon, brain stem, cerebellum and thoracic spinal cord were embedded in paraffin. Sections 10 micra thick were cut. The sections were stained by the hematoxylin and eosin, thionin, ammoniacal silver hydroxide; and Weil's myelin sheath methods.

RESULTS. Hyperchromic anemia was produced in all six of our dogs, as has been reported elsewhere (3). Four dogs sacrificed during the anemic phase of the experiment show multiple small hemorrhages throughout all portions of the nervous system. These petechiae are located predominantly in the grey sub-

¹ Research paper No. 591, journal series, University of Arkansas.



FIG. 3. Focal areas of destruction are found in the emerging roots of the spinal and cranial nerves, the significance of which is not established. A—Dorsal spinal nerve root. B—Cranial nerve root. H. & E. 100X.

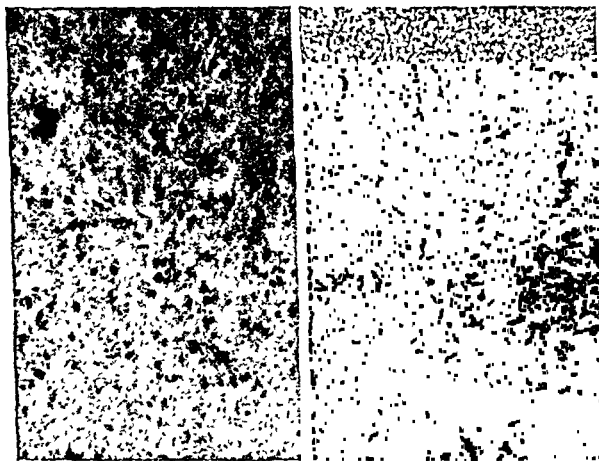


FIG. 4. Proliferation of glial cells and glial fibrous scar formation are characteristic features in the cerebral cortex of dogs which have recovered from the effects of choline and carbamyl-choline treatment. A—Section showing diffuse glial cell proliferation. B—Section showing a marked widening and dense fibrous replacement of the molecular zone of the cerebral cortex. The underlying cortical cells are markedly depleted in numbers. H. & E. 100X.

the cells. There is an early proliferation of the glial elements, more particularly the microglia cells. Neuronphagia is present in many cortical areas.

An occasional small area of beginning demyelination is seen in the brain tissue, but none are present in the spinal cord. The emerging nerve fibers of the



FIG. 2. Petechiae occur in the grey matter of the brain stem and spinal cord, but with less frequency than is seen in the cerebral cortex and cerebellum. A—Section of the spinal cord showing perivascular hemorrhages in the anterior grey horn. B—Section passing through the medulla showing small hemorrhages into one of the foci of nerve cells. Such appearances are common throughout the brain stem. The nerve cells show pyknosis. H. & E. 100X.

dorsal spinal nerve roots and certain of the cranial nerves show an occasional area of destruction (fig. 3).

In contrast to the experimental animals, only one or two small hemorrhages are seen in sections of the brains and spinal cords from the five untreated control animals. No other changes, other than a few scattered neurones showing acute degeneration, are observed.

ventricular walls shows in places an extensive perivascular gliosis and scar formation (fig. 5A). There appears to be a diffuse and generalized proliferation of glial cells throughout all portions of the nervous system. This is more pronounced in the forebrain (fig. 4A). The Purkinje cell layer of the cerebellum shows considerable disruption of its usual architecture. The tissue has a characteristic loose, fragmented appearance and there is a marked depletion in the number of Purkinje cells. Many of the remaining cells show degenerative changes (fig. 6). No significant changes are observed in sections through the brain stem.

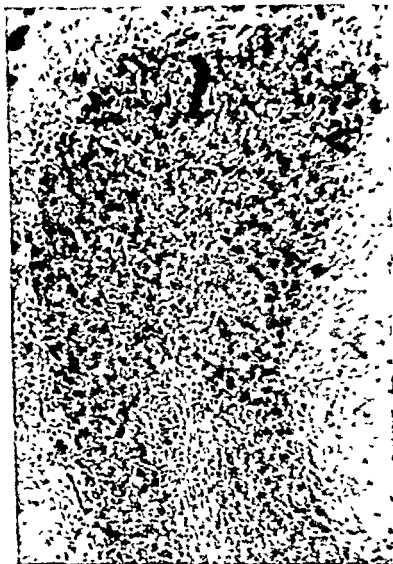


FIG. 6. Disruption of the Purkinje zone of the cerebellum with destruction of the Purkinje cells is a conspicuous process. It is suggested that this is the result of the hemorrhages which occur into this zone during the acute phase of the experiment. H. & E. 100X.

DISCUSSION. The nervous systems of dogs treated with choline and carbamylcholine show many changes not observed in untreated animals. The hemorrhagic phenomenon is the most conspicuous feature of the acute phase. It is interesting that this occurs primarily and almost exclusively in the grey substance. Petechiae occur frequently in the brain of man and animals in many disease processes and under experimental conditions. However, they usually occur predominantly in the white substance (4, 5). Brain purpura also is a frequent finding in pernicious anemia (4, 8, 9, 10). The hemorrhages may occur in either the gray or white matter. They apparently do not show a specificity for the grey matter such as is present in our experimental animals. However, on this point the literature is not clear.

In the present study allowance must be made for the possibility that some of these hemorrhages are the result of trauma incident to removal of the tissue.

The nervous systems of animals sacrificed after recovery from the experimentally produced anemia show no hemorrhages in any portions of the brain and spinal cord. Furthermore, sections from the spinal cords and spinal and cranial nerves show no significant alteration from those removed from the untreated animals, except for a slight hyperplasia of the ependymal lining of the central canal.

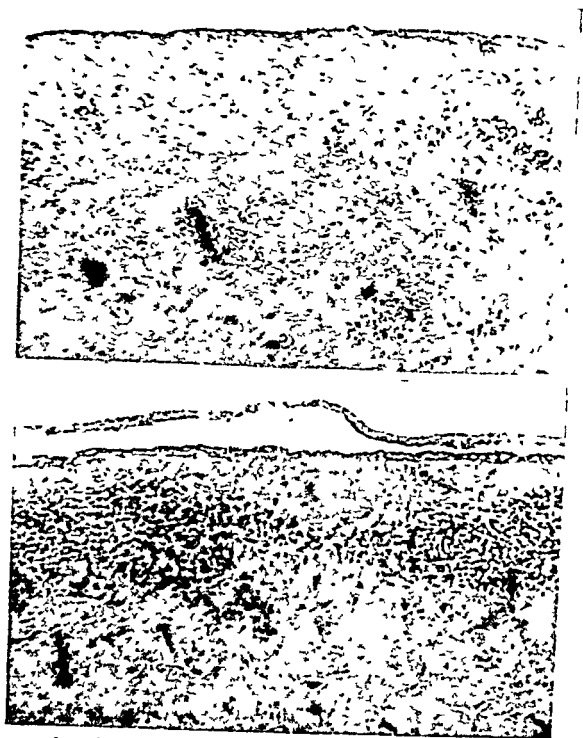


Fig 5 Perivascular gliosis and focal areas of glial cell proliferation are present in the brains of dogs which have recovered from the experimental anemia. A—Section of the brain adjacent to the ventricular wall showing a proliferation of glial cells and scarring about the blood vessels. This is not a perivascular round cell infiltration. B—Section of the molecular layer of the cerebral cortex showing glial nodule formation. H & E 100X.

A marked hyperplasia of the glial elements occurs in the cerebral cortex of animals which have recovered from medication. The molecular zone in certain regions is greatly widened at the expense of the underlying cellular layer. It now appears as a dense feltwork of glial fibers (fig. 4B). There is a marked diminution in the number of nerve cells adjacent to these areas. Other cortical areas show an extensive proliferation of the glial cells which form glial nodules. These occur along the molecular zone (fig. 5B). There is a hyperplasia of the ependymal lining of the brain ventricles. The brain substance adjacent to the

nervous system were also produced by these drugs. In the acute phase the lesions were characterized by multiple hemorrhages in the grey substance of the brain and spinal cord, and by a diffuse gliosis and acute neurone changes. After recovery from drug treatment, the lesions were characterized by perivascular gliosis, glial nodules, diffuse glial scar formation, and neurone depletion. The similarities and differences between these central nervous system changes and those described in pernicious anemia are mentioned. The possible etiological factors responsible for these changes are discussed briefly.

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The importance of this factor, however, is minimized by the fact that only very rarely is a hemorrhage found in the brain of a chronic experimental animal or in a control animal, although all animals were subjected to the same procedure for the removal of the brains and spinal cords.

A majority of the hemorrhages are small and perivascular in position. Exceptions are found in the cerebral cortex and cerebellum where larger ones occur. The small hemorrhages apparently are ultimately absorbed and leave no detectable residual scar. The larger ones may be responsible for the destruction seen in the Purkinje zone of the cerebellum and for the extensive perivascular scarring, gliosis, and nerve cell changes present in the cerebral grey substance of those animals allowed to recover from the effects of the drugs.

A direct toxic action of choline and carbamyl choline upon the nerve cells also must be considered as a possible etiological factor in the production of these central nervous system lesions. The proliferation of the ependymal cells of the ventricles and of the glia cells, together with the acute neurone changes, may well represent a response to the direct toxic action of these drugs. On the other hand, changes similar to these findings are present in human brains in pernicious anemia (4, 5, 7, 8, 9, 10).

Several theories have been advanced to explain the neuropathological changes in the brain and spinal cord in pernicious anemia (9, 11). Those based upon hemorrhage, vascular thromboses, and perivascular lymphatic stasis have been more or less discarded (6, 9, 10). The toxic theories, an example of which is Lurie's theory, are favored most at present. According to these theories a toxin is assumed to be liberated, perhaps from the gastro-intestinal tract, which acts directly upon the central nervous system and also independently upon the blood stream producing the anemia. The anemia in turn produces a disturbance in the cellular metabolism of nerve cells which secondarily enhances the destructive action of the hypothetical toxin.

We do not feel justified in postulating any such endogenous toxin to account for the production of the lesions found in this study. Neither can we deny its possible existence. The pathological changes present in the nervous system of our experimental animals might be explained on the basis of the combined direct action of the drugs and the hemorrhages. The anemia produced by the drugs was not severe enough to cause changes due to hypoxia.

The subacute combined degeneration of the spinal cord and the Lichtheim foci in the medullary substance of the brain which characterize the neuropathology of pernicious anemia are not present in our experimental animals. This discrepancy may be due to a species difference, to a time factor or to the fact that the hyperchromic anemia produced by the choline drugs may be unrelated to human pernicious anemia.

SUMMARY

Hyperchromic anemia was produced in six dogs by the regular daily administration of choline chloride and/or carbamyl choline. Definite changes in the

To eliminate the statistical variability of Ct exposures (5), the dosimetric technique was employed in the first experiments. The significance of alterations in observed mortality in the HMT protected animals was determined from the parameters of a previously established dosage-mortality curve (5).

After it had been demonstrated that increasing the HMT dose would protect against dosimetric exposures to high concentrations of phosgene, chamber exposures were used for the remaining experiments to permit better comparison with the earlier work. In these experiments, an effort was made to approximate roughly the relationship between concentration and dose of phosgene and the corresponding prophylactic HMT dose. Therefore, the exposure times were maintained at approximately 6 minutes, and the concentrations were altered accordingly.

Because Schultz *et al.* (2) and Gerard *et al.* (3) were unable to confirm previous reports (6) of the therapeutic efficacy of HMT administered after exposure to phosgene, only a few animals were treated with HMT injections after dosimetric exposure to an LD_{99.5}+ of phosgene. Since no beneficial effects were observed, the latter experiments were not continued.

METHODS: Eighty-three normal, healthy, unanesthetized mongrel dogs, weighing 4.5 to 18.6 kg., were used in these experiments. Diets consisting of ground horse meat, powdered milk, dried dog food, and water were permitted *ad libitum* before and after exposure. To ascertain the cause of death, autopsies were performed on all animals dying within the 96-hour observation period after exposure.

The treated animals were given stated doses (tables 1-5) of a 40% solution of hexamethylenetetramine (HMT) at various time intervals before exposure to phosgene. Ten treated and two untreated, control animals were exposed by the previously described technique (5) which permits measurement of the actual dose retained after inhalation. The prophylactic efficacy of the HMT in this group was evaluated by comparison with a previously determined dosage-mortality curve for dogs similarly exposed to phosgene (5). In the remaining experiments, groups of 4 or 5 treated and untreated animals were simultaneously exposed to different concentrations of phosgene for approximately 6 minutes in a dynamic chamber (7). The six-minute exposure period was used to allow sufficient time for re-equilibration of the phosgene concentration after placing the animals in the chamber.

Tables 1 to 5 present the pertinent experimental details for each series of animals, including phosgene dose and concentration, time of exposure, and dose and time of administration of HMT.

RESULTS: 1. *Prophylactic effect of 2.0 gm./kgm. doses of HMT in dogs dosimetrically exposed to a mean phosgene concentration of 6.72 mgm./l.* Nine of ten dogs which received 2.0 gm. of hexamethylenetetramine/kgm. survived doses of phosgene ranging from 2.43 to 7.39 mgm./kgm. The HMT was injected intravenously 1 to 1½ hours before exposure to a mean phosgene concentration of 6.72 mgm./l. One treated animal, which received a phosgene dose of 7.0 gm./kgm., died in 55 hours. Two controls, given phosgene doses of 3.39 mgm./kgm. and 4.06 mgm./kgm., died within 12 hours after exposure (table 1).

The dose of phosgene corresponding to 99.5% mortality for dogs exposed to this concentration (6) is 2.50 mgm./kgm. The P value (0.001) obtained by Bliss' direct chi square test (8) for the comparison of dosage-mortality data indicates that the reduction in mortality in the treated animals is highly significant.

THE PROPHYLACTIC EFFECT OF HEXAMETHYLENETETRAMINE IN DOGS EXPOSED TO HIGH CONCENTRATIONS OF PHOSGENE

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The report of Porembskii (1) that hexamethylenetetramine (HMT), administered before exposure, is highly effective in preventing diphosgene poisoning in mice stimulated other investigations (2, 3) which confirmed and extended his observations in several species. Porembskii tested prophylactic HMT doses of from 0.125 to 2.0 gm./kgm. and found that 33 of 33 mice given 2.0 gm./kgm. and 37 of 40 mice given 1.0 gm./kgm. survived exposure to diphosgene concentrations of 0.5 mgm./l. for 20 minutes, whereas the mortalities in untreated controls similarly exposed were 77% and 72.5%, respectively.

By administration of HMT to rats before exposure to 0.29 mgm./l. of phosgene for 13 minutes, Schultz *et al.* (2) were able to save 10 of 10 given 0.50 gm./kgm., 9 of 10 given 0.25 gm./kgm., and 8 of 10 given 0.125 gm./kgm. All 10 untreated controls died during the 72 hour observation period. This group also studied the prophylactic effect of different doses of a 40% HMT solution plus 50 cc of NaCO₃ administered by stomach tube to dogs two hours before exposure to different Ct's of phosgene. Most of the animals were exposed to 0.5 mgm./l. for 30 minutes. One untreated control was exposed with each group of 3 treated animals. If the data for Ct's from 13,300 to 17,900 γ -min./l. are combined, the survivals for the different HMT doses were as follows: 1/1 for 0.60 gm./kgm., 6/7 for 0.30 gm./kgm., 16/28 for 0.20 gm./kgm., 5/8 for 0.115 gm./kgm., 2/6 for 0.10 gm./kgm., 0/4 for 0.07 gm./kgm., and 2/18 for untreated controls.

In studying the relation between plasma HMT level and protection against phosgene poisoning, Schultz *et al.* (4) found that 8 of 9 dogs with HMT plasma levels which protected against given Ct's at lower phosgene concentrations died when exposed to the same Ct's with high concentrations and short exposures. These data, although on only 9 animals, suggested that the prophylactic efficacy of HMT is proportional not to Ct but to the concentrations of phosgene to which the animal is exposed.

Since with a non-persistent, irritating agent like phosgene accidental or combat exposure generally would be at high concentrations and for short times, it appeared desirable to determine if HMT could protect under these conditions. Furthermore, because it illustrates quantitatively the overwhelming of an artificial detoxification mechanism when a toxic agent enters the body at a very rapid rate, this relationship between phosgene dose and concentration and HMT action is also of theoretical interest.

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Five of 12 dogs in which 0.25 gm./kgm. doses of HMT were injected intravenously 16 to 67 minutes before exposure to 3.63 mgm./l. of phosgene for 6 minutes (mean Ct = 21,780 γ -min./l.) survived. All 8 simultaneously exposed, but untreated, controls died within 20 hours (table 4).

TABLE 3

Prophylactic effect of 0.5 gm of HMT/kgm in dogs exposed to a phosgene concentration of 3.89 mgm/l. in a dynamic chamber for 6* minutes*

NUMBER OF ANIMALS	IV. HMT DOSE	TIME BETWEEN HMT INJ AND EXPOSURE	AVERAGE CT*	MORTALITY FRACTION	TIME OF DEATH
	gm/kgm	minutes	γ -min/l		hours
6	0.5	10 to 53	23,310	2/6	19 to 45
13	controls		23,310	13/13	<18

* Mean chamber concentrations and exposure time are given. Actual Ct's varied from 22,800 to 25,800 microgram min/l.

TABLE 4

Prophylactic effect of 0.25 gm of HMT/kgm in dogs exposed to a phosgene concentration of 3.63 mgm/l in a dynamic chamber for 6* minutes*

NUMBER OF ANIMALS	IV. HMT DOSE	TIME BETWEEN HMT INJ AND EXPOSURE	AVERAGE CT*	MORTALITY FRACTION	TIME OF DEATH
	gm/kgm	minutes	γ -min/l		hours
12	0.25	16 to 67	21,780	7/12	20 to <96
8	controls		21,780	8/8	<21

* Mean chamber concentrations and exposure time are given. Actual Ct's varied from 20,760 to 23,580 microgram min/l.

TABLE 5

Prophylactic effect of 0.25 gm of HMT/kgm in dogs exposed to a phosgene concentration of 2.49 mgm/l in a dynamic chamber for 6.5* minutes*

NUMBER OF ANIMALS	IV HMT DOSE	TIME BETWEEN HMT INJ AND EXPOSURE	AVERAGE CT*	MORTALITY FRACTION	TIME OF DEATH
	gm/kgm	minutes	γ -min/l		hours
13	0.25	52 to 70	16,185	4/13	<24
3	control		16,185	2/3	<24

* Mean chamber concentration and exposure time are given. Actual Ct's varied from 14,980 to 18,200 microgram min/l.

Nine of 13 dogs given 0.25 gm./kg. doses of HMT 52 to 70 minutes before exposure to 2.49 mgm./l. of phosgene for 6 minutes (mean Ct = 16,185 γ -min./l.) survived. One of 3 untreated controls also survived (table 5).

The difference between the mortalities of the HMT-treated and of the untreated, control dogs for each series of chamber exposures is highly significant ($P \leq 0.001$) when the data are compared by Luykx' method for probabilities in small samples (9).

2. *Prophylactic effect of different doses of HMT in dogs exposed to phosgene concentrations from 2.49 to 7.98 mgm./l. in a dynamic chamber for six minutes. Ten of eleven dogs given 2 gm./kgm. doses of HMT intravenously about 1 hour before 5.9 minute exposures to an average phosgene concentration of 7.98 mgm./l.*

TABLE 1

Prophylactic effect of 2.0 gm. of HMT/kgm. in dogs exposed by the dosimetric method to an average phosgene concentration of 6.72 mgm./l.

INTRAVEN. HMT DOSE	TIME BETWEEN HMT INJ. AND EXPOSURE	MEAN CONTACT CONC. OF PHOSGENE*	RETAINED DOSE OF PHOSGENE	SURVIVAL TIME
gm./kgm.	minutes	mgm./l.	mgm./kgm.	hrs.
2.0	114	6.72	3.40	240+
2.0	94	6.88	3.20	240+
2.0	92	5.57	6.07	240+
2.0	90	7.31	3.49	240+
2.0	88	6.73	7.39	240+
2.0	80	7.07	2.44	240+
2.0	68	6.72	5.72	240+
2.0	65	6.51	7.06	ca.55
2.0	60	6.80	2.43	240+
2.0	60	6.90	3.29	240+
Control†		6.87	4.06	ca.12
Control†.		6.90	3.39	ca.12

* Mean contact conc. = actual conc. of phosgene to which animal is exposed in dosimetric gassing (1).

† Standard curve (1) for dogs exposed at this concentration indicates that dosage corresponding to 99.5% mortality = ca.2.50 mgm./kgm.

TABLE 2

Prophylactic effect of 2.0 gm. of HMT/kgm. in dogs exposed to a phosgene concentration of 7.98 mgm./l. in a dynamic chamber for 5.9* minutes*

NUMBER OF ANIMALS	I.V. HMT DOSE	TIME BETWEEN HMT INJ. AND EXPOSURE	AVERAGE Ct*	MORTALITY FRACTION	TIME OF DEATH
	gm./kgm.	minutes	γ -min./l.		hours
11	2.0	48 to 81	47,000	1/11†	<22
5	control		47,000	5/5	ca.4 hrs.

* Mean chamber concentration and exposure time are given. Actual Ct's varied from 46,400-47,800 microgram-min./l.

† Two animals died after 160 and 185 hours from pneumonia

(Ct's = 46,400 to 47,000 γ -min./l.) survived whereas, of five unprotected controls, four died in 3½ to 4 hours, and one died within 20 hours (table 2).

Four of six dogs similarly treated with 0.5 gm./kgm. doses of HMT 10 to 53 minutes before exposure survived exposure to a phosgene concentration of 3.89 mgm./l. for 6 minutes (mean Ct = 23,310 γ -min./l.). All 13 controls which were exposed with the treated animals died in less than 18 hours (table 3).

hour before 5.9 minute exposures to an average phosgene concentration of 7.98 mg./l. (mean Ct = 47,000 γ -min./l.) in a dynamic chamber survived. Five of five untreated controls died.

4. Four of six dogs which were injected with 0.5 grams/kg. doses of HMT 10-53 minutes before exposure to a phosgene concentration of 3.89 mg./l. for 6 minutes (mean Ct = 23,300 γ -min./l.) survived; 13 controls died in less than 18 hours.

5. After receiving 0.25 grams/kg. doses of HMT 60-70 minutes before exposure, 5 of 12 dogs survived a 6 minute exposure to 3.63 mg./l. of phosgene (mean Ct = 21,800 γ -min./l.) which killed all untreated animals; and 9 of 13 dogs survived a 6 minute exposure to 2.49 mg./l. of phosgene (mean Ct = 16,200 γ -min./l.) which killed 2 of 3 controls.

6. As the HMT dose was decreased, its prophylactic effect against phosgene poisoning diminished; however, at each HMT dosage the difference between the mortality of treated animals versus controls is statistically highly significant. The increase in mortality with decrease in HMT dose is suggestive of a quantitative relationship between the effective prophylactic dose of HMT and the concentration and dose of phosgene to which the animal is exposed.

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3. *Pathology.* All the animals which died within the 96-hour observation period following exposure exhibited clinical and post-mortem changes typical of phosgene poisoning (10). The survivors never developed more than slight moist rales, and, when sacrificed 10 or more days after exposure, exhibited no significant pulmonary pathology.

Moderate hematuria of one or two days duration occurred in approximately 10% of the dogs injected with 2.0 mgm./kgm. doses of HMT. It should be noted that no bicarbonate was administered with or after the HMT injections.

DISCUSSION: These data indicate that there is a definite relationship between the concentration and dose of phosgene and the HMT dosage which will protect dogs against an otherwise lethal exposure to that concentration. Thus, if the dose of HMT is increased to 2.0 gm./kgm. of body weight, significant protection is obtained against six-minute exposure to 7.98 mgm./l. of phosgene (mean Ct = 47,000 γ -min./l.) or dosimetric exposure to an LD99.5++ of phosgene at an average concentration of 6.72 mgm./l. As the dose of HMT is decreased, its protective effect against phosgene diminishes; e.g., a dose of 0.25 gm./kgm. affords less protection against exposure to phosgene for approximately 6 minutes at 3.63 mgm./l. than at 2.49 mgm./l. (cf. tables 4 and 5).

While HMT will apparently offer protection against exposure to high concentrations of phosgene for 6 minutes, the dosage required is very large. For example, if the HMT dose/kgm. effective against a particular phosgene concentration were the same in man and dog, a dose of 150 gm. of HMT would be required to protect a 75 kgm. soldier against a six-minute exposure to 8 mgm./l. of phosgene (Ct = 48,000 γ -min./l.). When the amount of bicarbonate needed to maintain an alkaline urine is considered, also, the practical difficulties become apparent. For less severe exposures, protection, of course, can be achieved by smaller doses.

The quantitative relationship between HMT and phosgene demonstrates how a specific detoxification mechanism may be overwhelmed by rapid administration of a toxic agent. This *in vivo* relationship between the doses of HMT and phosgene might be quantitatively defined with a larger series of animals dosimetrically exposed after receiving different amounts of HMT per kgm. body weight. For such studies better methods for the determination of HMT in blood and body tissues or fluids are needed.

SUMMARY

1. The prophylactic effect of hexamethylenetetramine (HMT), intravenously administered as a 40% solution in saline, was tested in normal, unanesthetized dogs exposed to several concentrations of phosgene by the dynamic chamber or the dosimetric technique.

2. Nine of ten dogs which received 2.0 grams of HMT/kg. of body weight 1 to 1½ hours before dosimetric exposure survived 2.43 to 7.39 mg./kg. doses of phosgene. (The LD99.5+ of phosgene for dogs similarly exposed to approximately the same concentration, 6.72 mg./l., is 2.50 mg./kgm.) Statistically, the reduction in mortality in the treated animals is highly significant.

3. Ten of eleven dogs which received 2 grams./kg. doses of HMT about one

TABLE 1

Showing marked individual differences in susceptibility to oral barbiturates in cats

NO CATS		PER CENT MORTALITY	NO CATS		PER CENT MORTALITY
[Seconal]			[Pentobarbital]		
	30 mg dose			100 mg dose	
5		0	5		0
5		0	5		0
5		0	5		0
5		0		100 mg dose	100
	50 mg dose		5		100
5		60		110 mg dose	80
5		100	5		80
5		100		125 mg dose	100
5		80	5		100
5		40	[Barbiturate of sigmodal]		
5		20		30 mg dose	
5		40	5		0
5		20	5		0
5		20	5		0
	60 mg dose			100 mg dose	0
5		40	5		0
5		40		110 mg dose	40
5		80	5		40
5		80		125 mg dose	100
	70 mg dose		5		40
5		80	5		80
5		80	5		80
5		20			80
5		80	[Pernoston]		
	80 mg dose			75 mg dose	
5		100	5		0
5		80		90 mg dose	0
4		75	5		0
	100 mg dose			110 mg dose	0
6		83	5		0
				125 mg dose	20
			5		20
				135 mg dose	100
			[Phenobarbital]		
				125 mg dose	0
			5		100
				160 mg dose	100
			5		40
				175 mg dose	100
			5		100
				185 mg dose	100
			5		100
				200 mg dose	100
			5		100

individual variations, as pointed out in the previous communication (1). Table 1 summarizes the results in groups of 5 cats as the experiments were carried out. The nearest approximation to the oral LD50 for the various compounds provided

COMPARATIVE STUDY OF SEVERAL BARBITURATES WITH OBSERVATIONS ON IRREVERSIBLE NEUROLOGICAL DISTURBANCES¹

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In a study of the pharmacology of secondary amyl-beta-bromallyl barbituric acid (barbiturate of signodal), it was observed that this compound produced effects within a few minutes after oral administration in cats, that the effects lasted for periods of days, and that in some cases neurological disturbances persisted after recovery from the narcosis and appeared to be irreversible (1). To what extent these effects differed from those of other barbiturates was not possible to determine from the literature. There are no satisfactory accounts of the speed of onset of barbiturate action after oral administration in cats. As regards the duration of action, different studies have used different criteria. There are also no accounts of irreversible damage of the central nervous system of the type we observed. The present study was planned to investigate these matters.

EXPERIMENTAL The following barbiturates were used (1) phenobarbital sodium (a representative of long-acting barbiturates), (2) pentobarbital sodium (a representative of short-acting barbiturates), (3) seconal sodium (similar to the barbiturate of signodal but without the bromine), (4) pernoston (similar to the barbiturate of signodal except for the secondary butyl in the place of the secondary amyl group), and (5) the barbiturate of signodal (sodium secondary amyl beta-bromallyl barbiturate)

All compounds were used in the form of their sodium salts, and were administered in a freshly prepared 10 per cent aqueous solution by stomach tube after the animal had been fasted for 24 hours. The solution was washed down with a few cc of water. The course of the effects was charted by frequent observations up to the time of death or for at least one week after apparently complete recovery.

The experiments with the various compounds were carried out concurrently in small groups of animals (usually 5 cats) in order to test the drugs under as nearly similar conditions as possible.

The appearance of ataxia was taken as an indication of the onset of action, its disappearance as an indication of complete elimination. Ataxia as a criterion does not strictly represent either but it is a clear cut symptom and is near enough to both to be useful for comparative purposes.

RESULTS. Doses. In order to compare the course of action of the various compounds, it was necessary to use pharmacologically comparable doses. Accordingly, an attempt was made to determine the LD₅₀ of each compound, and to compare the course of the survivors. It was not possible to fix the oral LD₅₀ with precision unless large numbers of cats were to be used, because of the marked

¹ This study was aided by a grant from Riedel de Haen, Inc.

approximately 8 per cent of cases of irreversible damage of the central nervous system among the 102 survivors of varying large doses of the barbiturate of sigmodal. It should be noted that while most of the cases occurred after very large doses, one animal developed the permanent damage after an oral dose of 30 mg. per Kg., which is in the upper range of the amounts which have been used in humans. The disturbances varied from animal to animal, and involved disorders of reflexes, posture, and gait. The variety of changes suggested diffuse and generalized involvement of the central nervous system. The damage is apparently not due solely to very deep or prolonged narcosis. The details of the behavior of the 8 animals are summarized in table 3.

Histological sections. The histological study was made by Dr. Lewis D. Stevenson of the Department of Pathology. Approximately 170 sections were made in the case of 5 of these cats through various levels from the cortex down through and including the spinal cord. With the use of appropriate stains, the tissues were examined for changes in cells, myelin sheaths, meninges, vessels, and fat. The histological changes varied greatly. There were no generalized inflammatory or vascular lesions. There were isolated and occasional hemorrhages. There was no increase in the fat in the cortical cells. The myelin sheaths were normal. The most conspicuous abnormality was found in the Purkinje cell layer of the cerebellum; the cells showed degeneration, and were reduced in number so that in some areas the layer was completely destroyed.

The potentiality for irreversible damage of the central nervous system in cats seems to be especially high in the case of secondary amyl-beta-bromallyl barbituric acid (2). It did not occur with seconal (120 cats), pentobarbital (30 cats), or phenobarbital (30 cats). There was one case among 30 cats which received pernoston. This animal received an oral dose of 125 mg. of pernoston per Kg. It developed deep narcosis which lasted 36 hours. From the fourth day on, the animal appeared alert and apparently normal, but when it walked about, peculiar behavior was in evidence; the gait was serpent-like; there was a tendency to lurch forward; there was periodic arching of the back; it assumed awkward postures; there was persistent pilomotor stimulation. There was some tendency to improve, but the abnormalities were still present 113 days after the dose, when the animal was sacrificed and the brain sections prepared for histological examination. The main change was the reduction of the number of the Purkinje cells of the cerebellum as in the case of the other animals.

As the present data stand, there is indication that the bromallyl group bears a responsibility for the irreversible change in the central nervous system. It may be noted in figure 1 that seconal, which did not produce the effect, has the same structure as the barbiturate of sigmodal but without the bromine atom, and that pernoston, which possesses the bromallyl group of the barbiturate of sigmodal but has the secondary butyl group in place of the secondary amyl group, also has some potentiality for the peculiar effect on the central nervous system.

The high incidence of permanent injury of the central nervous system in cats raised the question of species susceptibility. The actions of the barbiturate of sigmodal were, therefore, explored in the dog for evidence of this change. The

by the data is: seconal, 50 mg. per Kg.; pentobarbital, 100; signodal, 110; pernoston, 135; phenobarbital, 175. All animals which received within approximately ± 20 per cent of these doses were included in the data describing the course of action.

Table 2 presents a summary of the results bearing on the speed of onset of effects, the duration of the maximum effects, and the time to recovery in the case of comparable doses of the 5 compounds. Because of the marked individual variations within any group, the differences between the averages are of doubtful significance. Absorption of all 5 compounds is very rapid. Effects are in evidence within about 5 minutes after the oral administration of a dose. They develop rapidly and the maximum level of narcosis is present within about 30 minutes. It continues without conspicuous reduction for about 18 hours when signs of recovery begin to appear. Ataxia, which is the first unmistakable sign of absorption, disappears in a period of 2 to 3 days. With respect to the foregoing factors, seconal, pentobarbital, the barbiturate of signodal, and pernoston

TABLE 2
Course of action of oral barbiturates in cats

DRUG	NO OF CATS	AVERAGE			
		Onset of ataxia	Time to maximum effect	Duration of maximum effect	Time to disappearance of ataxia
		min	min	hrs	hrs
Seconal	60	3 (1-5)	29 (7-56)	19 (3-62)	61 (29-120)
Pentobarbital	30	4 (2-8)	13 (5-32)	16 (6-40)	47 (28-96)
Signodal	30	4 (1-10)	17 (8-91)	21 (9-54)	61 (28-95)
Pernoston	20	5 (2-10)	14 (8-20)	12 (4-17)	72 (47-119)
Phenobarbital	25	8 (3-23)	45 (5-225)	23 (2-48)	239 (175-312)

show no outstanding differences. In the case of phenobarbital, the time to the disappearance of ataxia is approximately 3 times as long.

A state of unrest of varying degrees ranging from mild irritability to violent excitement occurred in some animals during the development of narcosis or during the recovery phase and sometimes during the height of the action of the barbiturates. Some degree of excitement occurred in from 31 to 83 per cent of groups of animals with all 5 compounds, unrest at one time or another being present in approximately half of the cats used in the study. The various compounds showed no significant difference with respect to this phenomenon.

Irreversible damage of the central nervous system. In the previous study (1), 165 cats received very large doses of the barbiturate of signodal alone or in combination with antipyrine. Of the 74 animals which survived, 6 developed a permanent disturbance of the central nervous system persisting long after the narcotic effect had disappeared. In the present study 45 additional cats received the barbiturate of signodal, and in this group 2 cats developed similar irreversible damage of the central nervous system. This makes an incidence of

TABLE 3—Continued

CAT NO.	DOSE (MG./KG.)		ROUTE	EFFECTS
	Barbi- turate	Anti- pyrine		
84	110	0	Oral	Deep narcosis for 36 hrs. <i>Day 4</i> : Normal. <i>Day 8</i> : Landing reflex abnormal; spastic gait. <i>Day 9</i> : High steppage gait; otherwise normal. <i>Day 12</i> : Tends to reel; high steppage gait; otherwise normal. <i>Day 110</i> : Above condition unchanged; sacrificed; no gross pathology.
37	125	0	Oral	Deep narcosis for 30 hrs. <i>Day 6</i> : Slightly spastic gait; abnormal landing reflex; reflexhy perexcitability. <i>Day 8</i> : Spasticity at beginning of walking. <i>Day 13</i> : Spasticity and catatonia (maintains bizarre postures). <i>Day 39</i> : Same as above; otherwise normal; sacrificed.

* Antipyrine was used in these experiments taken from the study on "Sigmodal" (1), a trade name for a solution containing a mixture of antipyrine and secondary amyl beta-bromallyl-barbituric acid.

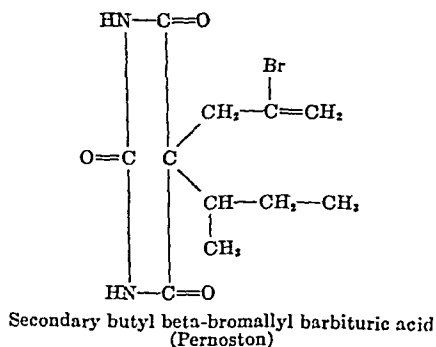
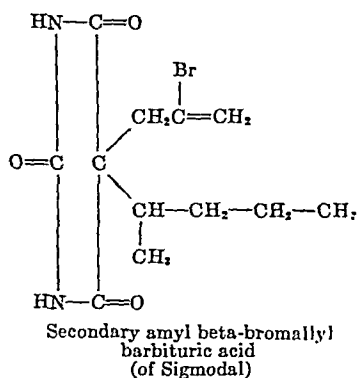
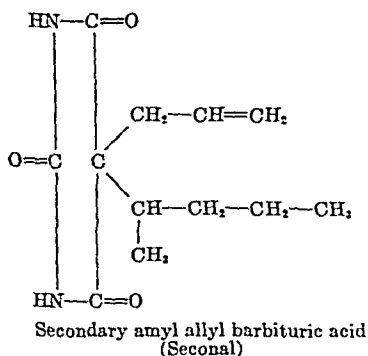


FIG. 1

TABLE 3

*Irreversible damage of the central nervous system in cats after SABBB
and the mixture with antipyrine*

CAT NO.	DOSE (MG./KG.)		ROUTE	EFFECTS
	Barbi- turate	Anti- pyrine		
172	30	0	Oral	Deep narcosis for 2 hrs. <i>Day 2</i> : Alert; no ataxia; tremors of extremities, especially hind limbs; clonus of limbs. <i>Day 3</i> : Looks well; takes food normally; gait normal; lands awkwardly when jumps; clonus. <i>Day 20</i> : as above. <i>Day 105</i> : Convulsion; otherwise same. <i>Day 182</i> : Clonus increased; sacrificed.
178	100	0	Rectal	Light narcosis for 1 hr. <i>Day 3</i> : Looks well; takes food normally; slight rotation of head, flexion of neck to the right and veers toward right when walking. <i>Day 4</i> : Abnormality of head posture increased; walks with lurch to the right. <i>Day 20</i> : Looks well; eats; purrs; abnormal head posture with lurching and veering to right when walking persists. <i>Day 216</i> : Condition same; sacrificed.
78	125	0	Oral	Deep narcosis for 36 hrs. <i>Day 4</i> : Awake; spastic; holds awkward posture (cataleptic); over - and - under - shooting; loss of initiative; responds to pain with "sham rage"; loss of placement reaction. <i>Day 7</i> : Drinks milk and water; as above; spastic, prancing, ataxic gait. <i>Day 10</i> : Nutrition good; takes food eagerly; otherwise same; sacrificed for histological study.
141	150	0	Oral	Narcosis for 2 days. <i>Day 3</i> : Reflex hyperexcitability; unable to sit up, but alert. <i>Day 6</i> : Spastic; ataxic gait; seizures of excitement. <i>Day 9</i> : Refuses food; weak; unable to walk; 40% weight loss; parenteral glucose and saline given daily. <i>Day 23</i> : Eats well; sits; body sways. <i>Day 29</i> : Eats well; walks; marked ataxia; high steppage gait; stands swaying on broad base; placement reflexes absent; purrs. <i>Day 120</i> : Weight exceeds original; above neurological defects remain. <i>Day 266</i> : Condition same; sacrificed.
27	50	275*	Intrave- nous	Deep narcosis for 3 days. <i>Day 4</i> : Awake; ataxic gait. <i>Day 6</i> : More marked ataxia. <i>Day 8</i> : Weaker; does not stand. <i>Day 9</i> : Same, but drinks milk; sacrificed; no gross pathology.
125	100	500*	Oral	Deep narcosis. <i>Day 3</i> : Narcosis lighter; marked muscular spasticity. <i>Day 5</i> : Awake; almost convulsive; runs about wildly with ataxia. <i>Day 6</i> : Does not stand; marked spasticity; strychnine-like convulsion. <i>Day 8</i> : Placement reflexes absent; twitching. <i>Day 11</i> : Body sways while sitting; ataxic gait; eats and drinks; same condition to day 34. <i>Day 35</i> : Found dead in morning.

and disappearance of ataxia in 2 to 3 days. The onset in the case of phenobarbital is somewhat slower, and the time for disappearance of ataxia is about 3 times as long.

3. The duration of action of the barbiturate of sigmodal in the dog is somewhat shorter than in the cat.

4. Large doses of secondary amyl-beta-bromallyl barbituric acid (barbiturate of sigmodal) produce an irreversible damage of the central nervous system resulting in motor, postural and reflex abnormalities in about 8 per cent of cats, but not in dogs.

5. Large doses of secondary butyl-beta-bromallyl barbituric acid (pernoston) also possess this action on the central nervous system to some degree in cats.

6. This action on the central nervous system of the cat was not observed with several other compounds free of the bromallyl group, namely, seconal, pentobarbital, and phenobarbital.

7. In view of the species differences, the large doses of the barbiturate which were used to produce these central nervous system effects, and the absence of reports of such effects from their therapeutic use, these observations in the cat may not be applicable to the use of these barbiturates in humans, at least in the range of hypnotic doses.

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compound was given in 10 per cent solution by stomach tube after food was withheld for 24 hours. Additional observations relating to the course of effects, similar to those made in the case of the cats, were recorded.

The results in 47 dogs are summarized in table 4. There was no evidence of the permanent functional damage of the central nervous system which was seen in the cat. The dogs either died or recovered completely.

With respect to the course of effects, the behavior of the dog appears to be substantially similar to that of the cat. Ataxia appeared in about 5 minutes. The full effects developed within about one-half to three-quarters of an hour. The duration of action was somewhat shorter, an average of about 6 to 11 hours for the maximum depression with different doses, and 1 to 2 days for the disappearance of the ataxia. The duration of narcosis in the dog was similar to that reported by Hazleton, Koppanyi, and Linegar (3). A similar high degree of

TABLE 4
Sodium secondary amyl-beta-bromallyl barbiturate in dogs

NO ANY MALES	DOSE	ONSET		TIME TO MAXI MUM EFFECT		DEGREE OF EFFECT		DURATION OF MAXIMUM EFFECT		TIME TO RECOVERY		RECov- ERY	EX CITE MENT*
		Average	Range	Average	Range	Average	Range	Average	Range	Average	Range		
	mg / Kg	min	min	min	min			hrs	hrs	hrs	hrs		
28	30	5 6	2 to 11	43 7	4 to 184	2 4+	1+ to 5+	6 6	0 5 to 19	24 6	8 to 280	100%	57%
10	70	5 5	2 to 9	44 0†	6 to 102	2 6+	1+ to 5+	11 1	3 to 25	21 8†	14 to 28	90%	60%
9	80	4 6	2 to 8	24	14 to 37	4+	1+ to 5+	6 8	0 25 to 15	47§	30 to 71	56%	22%

* Unrest and/or excitement usually before or after narcosis, sometimes during narcosis

† One died 1 5 hrs after dose (not included in average)

‡ One dog took 321 min to develop full narcosis (not included in average)

§ Four dogs died in average of 1 25 hrs after dose (not included in average)

1+ (alert, able to stand, but unable to walk)

2+ (alert, but unable to stand)

3+ (narcosis, muscular relaxation, but reacts to painful stimuli)

4+ (deep narcosis, only corneal reflex present)

5+ (deep narcosis, can elicit no reflexes)

individual variation was in evidence. It may be noted that animals showing negligible depression as well as those showing deep narcosis were found in both the 30-mg. group and the 80-mg. group.

SUMMARY

1. The approximate oral LD50 for several barbiturates in the cat were as follows: seconal, 50 mg. per Kg.; pentobarbital, 100; the barbiturate of sigmodal, 110; pernoston, 135; phenobarbital, 175. Attention is called to the extreme individual variation which limits the precision of the LD50 values.

2. The courses of action after oral administration of the approximate LD50 dose in the cat are substantially similar for seconal, pentobarbital, the barbiturate of sigmodal, and pernoston, namely, onset of ataxia in about 5 minutes, maximum narcosis in about 30 minutes, duration of the full effects for about 18 hours,

van Dongen (7), Martin and Lissák (8)). According to van Dongen (7) and Martin and Lissák (8) 933F counteracts also the effect of electric stimulation on the heart but this strictly "sympatholytic" action requires larger doses than the "adrenolytic" one (Martin and Lissák) although artificially administered sympathin, obtained from hearts or from the anterior eye chamber, is easily counteracted by 933F (Lissák (9)).

Benzylimidazoline (Priscol) whose adrenolytic action was described in 1939 by Hartmann and Isler (10) has been studied extensively throughout Europe during the war years, however with only scanty attention paid to its action on the heart. On the isolated frog heart no antagonism against epinephrine could be demonstrated (Müller (11)). Effects on the normal electrocardiogram are controversial (Weitzmann (12), Francaviglia and Turchetti (13)). The pulse rate in humans was found diminished for about 45 minutes following intravenous injection of 0.15 to 0.16 mgm./kg. of Priscol (Francaviglia and Turchetti (13)).

The newest and apparently by far the most potent adrenolytic drug is dibenzyl- β -chloroethylamine hydrochloride (Dibenamine hydrochloride) as reported by M. Nickerson (14) at the Federation Meeting at Atlantic City in March 1946. In a dosage of 20 mgm./kg. it prevented epinephrine-induced cardiac irregularities, ventricular tachycardia and cardiac death in cyclopropane-anesthetized dogs. The epinephrine dose used was 10 γ /kg.

METHOD. Mature white rats (200-300 gm.) were injected intraperitoneally with 10 γ /gm. (in a few instances 20 γ /gm.) each of 933F, Priscol and Dibenamine hydrochloride.² In several control groups the animals were killed (by crushing the cervical spine) after varying time intervals following injection of these drugs and the hearts were immediately analyzed for their content in epinephrine-like adsorbable chromogens ("AC") (see Raab 15 a, b).

A second control series of rats was injected either intraperitoneally or intramuscularly with 10 γ /gm. of epinephrine, a dose which proved fatal in all instances in a matter of minutes.

Finally, several groups of animals received the same dose of epinephrine after having been pretreated at different time intervals with the aforementioned adrenolytic drugs. Unless death by drug action occurred prematurely, the pretreated rats of these latter groups were killed after a number of minutes following injection of epinephrine which was equal to the number of minutes of survival of corresponding epinephrine-injected control animals. Thus the time elapsed between epinephrine injection and death was practically the same in the epinephrine controls and in the rats pretreated with adrenolytic drugs.

933F and Priscol were injected in Ringer solution. Dibenamine hydrochloride was first dissolved in a small amount of propylene glycol and subsequently diluted with bicarbonate-free Ringer solution which had been slightly acidified with hydrochloric acid.

Immediately after death the hearts were removed, washed, dried superficially, weighed, ground with Ottawa sand in 10% trichloroacetic acid, the extracts filtered and the filtrates analyzed with the method of Shaw as modified by one of us (R. (15 a)).

This method and its specificity for epinephrine and related sympathomimetic catechol compounds have been discussed elsewhere (Raab (15 a, b)). Proof of its specificity has been further amplified by its application to adrenal tissue, sympathetic ganglia and fibres

² 933F was kindly provided by Dr. Louis Goodman, then Professor of Pharmacology and Physiology at the University of Vermont, Priscol by Dr. Ernst Oppenheimer of Ciba and Dibenamine hydrochloride by Dr. William Gump of Givaudan-Delawanna Chemical Works.

PROTECTIVE EFFECT OF ADRENOLYTIC DRUGS AGAINST FATAL MYOCARDIAL EPINEPHRINE CONCENTRATIONS¹

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The term "adrenolytic" is commonly being used in reference to drugs, the action of which abolishes or reverts certain pharmacodynamic effects of injected or secreted epinephrine (adrenalin), regardless of the mode of this counteraction. Some authors distinguish between "adrenolytic" action as above defined and "sympatholytic" action, the latter interfering with the effect of sympathetic stimulation and of the resulting intracellular discharge of sympathin from the sympathetic nervous endings into their respective effector cells. Both actions do not necessarily coincide, at least not as far as their intensity is concerned.

Most of the studies of adrenolytic and sympatholytic drugs are dealing with the responses of blood pressure, intestinal tonus, salivary secretion, etc. while only a comparatively few are concerned with cardiac reactions.

The recent discovery of some new adrenolytic drugs and the arising question of their possible usefulness in human cardiac pathology suggested the experiments described below which were intended to determine their efficacy in protecting against acute fatal cardiac failure through epinephrine intoxication of the heart.

Fourneau's diethylaminomethylbenzodioxane (883F) and piperidomethyl-3-benzodioxane (933F) have been known for more than a decade.

According to Sterne, Bovet and Lenoir (1), 883F in a dosage of 5 to 10 mgm./kg. moderately counteracts the electrocardiographic features resulting from ligation of the coronary arteries in the dog, diminishes adrenalin-induced and other tachycardias in man and adrenalin-induced extrasystoles, sometimes normalizes pathological ventricular complexes and aggravates existing auriculo-ventricular block. It reduces the heart rate in general but has no effect on the refractory period of the heart nor on the shape of the normal electrocardiogram. It does not counteract the fibrillation-producing effect of electric stimulation. According to Shen the "adrenalin-chloroform-syncope" is counteracted by 883F and in angina pectoris the drug has been found therapeutically useful (Clerk, Sterne and Lenoir (2)).

Reports on the cardiac effects of 933F are somewhat contradictory: Hill and Myers (3) found it to be but little effective against epinephrine-induced or sympathetic stimulation of the cat heart. According to deVleeschhouwer (4) the epinephrine-tachycardia in dogs is not impaired by 933F. On the other hand, the effects of epinephrine on the frog heart and on the isolated mammalian heart were found to be diminished or suppressed by 933F regarding inotropic action and the occurrence of heterotopic rhythms (Shen (5), Jourdan and Froment (6),

¹This study was aided by a grant from the John and Mary R. Markle Foundation.

933F and Dibenamine hydrochloride, on the other hand (the latter both within 1 hour and 24 hours; table 2), were followed by a distinct average increase of the heart AC. In about half of the animals the myocardial AC reached even levels above 2000 color units per gram which would have been fatal under normal circumstances. Normally, any myocardial AC concentration above 1900 col.un./gm. results in fatal cardiac failure (Raab (15a, 20)). None of the rats treated with the adrenolytic drugs alone showed any signs of discomfort.

Epinephrine (10 γ /gm) alone injected, intraperitoneally or intramuscularly, proved rapidly fatal in all animals although the average duration of survival was, for unknown reasons, somewhat longer than it had been in other series with

TABLE 2
Injection of epinephrine alone (10 γ /gm.)

DATE	MODE OF INJ.	WT. OF HEART (% OF BODY WT.)	AC (COL.UN./GM. HEART)	D.S.R.	TIME FROM INJ. TO DEATH	MODE OF DEATH	REMARKS
					min.		
3/26/46	i.p.	0.28	4,344	1.54	10	spontan.	Usual features*
3/28/46	i.p.	0.29	5,808	1.31	5	spontan.	Usual features
4/ 6/46	i.p.	0.29	2,782	1.11	25	spontan.	Usual features
4/ 6/46	i.p.	0.29	2,643	1.19	17	spontan.	Usual features
4/ 8/46	i.p.	0.29	3,356	1.15	9	spontan.	Usual features
4/ 8/46	i.p.	0.34	15,041	1.43	8	spontan.	Rapidly into coma
4/ 9/46	i.p.	0.33	4,229	2.00	12	spontan.	Usual features
4/ 9/46	i.p.	0.29	2,470	1.18	7	spontan.	Usual features
4/11/46	i.m.	0.39	2,267	1.23	28	spontan.	Usual features
4/11/46	i.m.	0.31	8,764	1.36	9	spontan.	Usual features
(1941/46)	(none)	(0.33)†	(1,268)†	(1.11)†	—	—	—

* Dyspnea, weakness, gasping, convulsions, pulmonary edema with bloody foam at nostrils.

† Average of readings obtained in 143 untreated control rats.

smaller doses (table 5). Death occurred after the usual epinephrine-induced sequence of weakness, dyspnea, gasping, convulsions and appearance of bloody foam at the nostrils. The hearts were dilated, the blood was dark. All myocardial AC concentrations were well within the fatal range. The average d.s.r. of 1.35 indicated the presence of considerable amounts of undeteriorated epinephrine participating in the total mass of epinephrine-like AC found in the heart.

After pretreatment with 933F the same dose of epinephrine caused death only in 44% of the animals, preceded by the usual features. The surviving animals showed hardly any discomfort. The average myocardial AC concentration was about the same as after 933F alone without epinephrine but the d.s.r. rose to 1.44. The highest tolerated and the lowest fatal AC concentration were not far above the critical level of 1900 col.un./gm (table 6).

Priscol reduced the mortality from epinephrine to 10%. The one succumbing

and by quantitative correlation of the colorimetric readings with the biological effects of dialyzates obtained from these tissues (Raab (15 c)).

The term AC (adsorbable chromogens) will be used, as in previous publications, to designate the total colorimetric readings. One color unit corresponds to 0.001 γ of epinephrine. The term d.s.r. (denominator of specific ratio, see Raab (15 a, pages 194, 196, 197)) permits certain conclusions regarding the qualitative composition of the material detected: the higher above 1.00 the d.s.r. the greater the participating quantities of unaltered epinephrine.

TABLE 1

Injection of adrenolytic drugs alone (10 γ /gm. each, i.p.). All animals killed

DRUG	DATE	WT. OF HEART (% OF BODY WT.)	AC* (COL. UN./GM. HEART MUSCLE)	D.S.R.†	TIME FROM INJ. TO DEATH
933F	6/1/46	0.29	1,800	1.00	30 min.
	6/1/46	0.30	2,931	?	30 min.
	6/1/46	0.27	2,551	0.97	30 min.
	6/1/46	0.26	1,659	?	30 min.
	6/1/46	0.26	1,613	1.00	30 min.
Priscol	5/24/46	0.31	1,687	1.00	30 min.
	5/24/46	0.34	1,366	1.10	30 min.
	5/24/46	0.29	1,636	1.10	30 min.
	5/24/46	0.29	1,404	1.04	30 min.
	5/24/46	0.28	1,048	1.16	30 min.
	5/24/46	0.38	1,346	1.07	30 min.
Dibenamine hydro- chloride	3/26/46	0.27	1,894	1.05	1 hour
	3/28/46	0.30	2,447	1.23	1 hour
	5/25/46	0.32	1,816	1.10	1 hour
	5/25/46	0.32	2,077	1.00	1 hour
	5/25/46	0.29	1,818	1.02	1 hour
	5/25/46	0.32	2,433	1.04	24 hours
	5/25/46	0.36	2,028	1.13	24 hours
	5/25/46	0.37	1,827	1.03	24 hours
	5/25/46	0.32	2,200	?	24 hours
	5/25/46	0.29	1,707	1.04	24 hours
(None)	(1941/6)	(0.33)‡	(1,268)‡	(1.11)‡	—

* The term "AC" stands for adsorbable chromogens (see text, p. 269).

† The term "d.s.r." stands for denominator of specific ratio (see text, p. 269).

‡ Average of readings obtained in 143 untreated control rats.

RESULTS. First, the adrenolytic drugs under consideration were kept in solution together with epinephrine in vitro for 30 minutes in mutual proportions equivalent to the dosage which had been found sufficient to abolish the vasoconstrictor effect of epinephrine in vitro (1 part epinephrine to 300-500 parts of the drugs). Neither the chromogenic properties of epinephrine, nor the d.s.r., nor the adsorbability of epinephrine by $\text{Al}(\text{OH})_3$ were appreciably altered.

Intraperitoneal injection of Priscol per se did not affect the basic myocardial AC concentration as compared with 143 older and recent controls (table 1).

933F and Dibenamine hydrochloride, on the other hand (the latter both within 1 hour and 24 hours; table 2), were followed by a distinct average increase of the heart AC. In about half of the animals the myocardial AC reached even levels above 2000 color units per gram which would have been fatal under normal circumstances. Normally, any myocardial AC concentration above 1900 col.un./gm. results in fatal cardiac failure (Raab (15a, 20)). None of the rats treated with the adrenolytic drugs alone showed any signs of discomfort.

Epinephrine (10 γ /gm) alone injected, intraperitoneally or intramuscularly, proved rapidly fatal in all animals although the average duration of survival was, for unknown reasons, somewhat longer than it had been in other series with

TABLE 2
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					min.		
3/26/46	i.p.	0.28	4,344	1.54	10	spontan.	Usual features*
3/28/46	i.p.	0.29	5,808	1.31	5	spontan.	Usual features
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4/ 6/46	i.p.	0.29	2,643	1.19	17	spontan.	Usual features
4/ 8/46	i.p.	0.29	3,356	1.15	9	spontan.	Usual features
4/ 8/46	i.p.	0.34	15,041	1.43	8	spontan.	Rapidly into coma
4/ 9/46	i.p.	0.33	4,229	2.00	12	spontan.	Usual features
4/ 9/46	i.p.	0.29	2,470	1.18	7	spontan.	Usual features
4/11/46	i.m.	0.39	2,267	1.23	28	spontan.	Usual features
4/11/46	i.m.	0.31	8,764	1.36	9	spontan.	Usual features
(1941/46)	(none)	(0.33)†	(1,268)†	(1.11)†	—	—	—

* Dyspnea, weakness, gasping, convulsions, pulmonary edema with bloody foam at nostrils.

† Average of readings obtained in 143 untreated control rats.

smaller doses (table 5). Death occurred after the usual epinephrine-induced sequence of weakness, dyspnea, gasping, convulsions and appearance of bloody foam at the nostrils. The hearts were dilated, the blood was dark. All myocardial AC concentrations were well within the fatal range. The average d.s.r. of 1.35 indicated the presence of considerable amounts of undeteriorated epinephrine participating in the total mass of epinephrine-like AC found in the heart.

After pretreatment with 933F the same dose of epinephrine caused death only in 44% of the animals, preceded by the usual features. The surviving animals showed hardly any discomfort. The average myocardial AC concentration was about the same as after 933F alone without epinephrine but the d.s.r. rose to 1.44. The highest tolerated and the lowest fatal AC concentration were not far above the critical level of 1900 col.un./gm (table 6).

Prisol reduced the mortality from epinephrine to 10%. The one succumbing

TABLE 3

Injection of adrenolytic drugs, followed by injection of epinephrine

(The dosage of all preparations was 10 γ /gm., except in the first four experiments with Dibenzamine hydrochloride when 20 γ /gm. were given.) All adrenolytic drugs were administered intraperitoneally.

DRUG	DATE	MODE OF INJ. OF EPINE- PHR.	WT. OF HEART (% OF BODY WT.)	AC (COL. UN. PER CM. HEART MUSCLE)	D.S.R.	TIME FROM DRUG INJ. TO EPINEPHR. INJ.	TIME FROM EPINE- PHR. INJ. TO DEATH	MODE OF DEATH	REMARKS
							min.		
933F	4/ 6/46	i.p.	0.29	3,193	1.13	30 min.	25	S.†	Usual features
	4/ 6/46	i.p.	0.39	3,527	1.92	30 min.	15	S.†	Usual features
	4/ 8/46	i.p.	0.26	2,091	1.32	30 min.	9	K.	Sl. dyspnea
	4/ 8/46	i.p.	0.27	1,715	1.14	30 min.	8	K.	No symptoms
	4/ 9/46	i.p.	0.33	1,329	2.00	30 min.	12	K.	No symptoms
	4/ 9/46	i.p.	0.28	810	2.20	30 min.	7	K.	No symptoms
	4/11/46	i.m.	0.33	3,107	1.19	30 min.	18	S.	Usual features
	4/11/46	i.m.	0.27	2,134	1.19	30 min.	9	K.	Dyspnea
	4/12/46	i.m.	0.26	1,901	1.03	30 min.	13	K.	No symptoms
	4/12/46	i.m.	0.30	2,943	1.26	30 min.	15	S.	Usual features
Priscol	4/ 8/46	i.p.	0.30	2,359	1.21	30 min.	9	K.	Sl. weakness
	4/ 8/46	i.p.	0.36	2,021	1.53	30 min.	8	K.	Sl. weakness
	4/ 9/46	i.p.	0.27	1,773	1.40	30 min.	7	K.	Sl. weakness
	4/ 9/46	i.p.	0.30	2,903	1.07	30 min.	12	K.	No symptoms
	4/11/46	i.m.	0.31	2,656	1.16	30 min.	23	K.	Dyspnea
	4/11/46	i.m.	0.26	1,847	1.23	30 min.	9	K.	No symptoms
	4/12/46	i.m.	0.25	1,845	1.07	30 min.	13	K.	Weakness
	4/12/46	i.m.	0.29	2,479	1.02	30 min.	30	S.	Usual features
	4/12/46	i.m.	0.25	2,062	1.10	30 min.	13	K.	No symptoms
	4/12/46	i.m.	0.26	1,532	1.10	30 min.	13	K.	No symptoms
Dibenzamine hydrochloride	3/26/46	i.p.	0.26	9,325	1.11	1 hour	10	K.	Sl. weakness
	3/28/46	i.p.	0.28	6,585	1.19	1 hour	5	K.	Sl. weakness
	4/ 6/46	i.p.	0.29	3,341	1.15	1 hour	25	K.	Sl. weakness, dyspnea
	4/ 6/46	i.p.	0.26	1,033	2.30	1 hour	17	K.	No symptoms
	4/ 8/46	i.p.	0.30	5,567	2.59	1 hour	9	K.	Weakness
	4/ 8/46	i.p.	0.27	6,830	1.46	1 hour	8	K.	Weakness, sl. dyspnea
	4/ 9/46	i.p.	0.28	1,331	1.13	1 hour	12	K.	Sl. weakness
	4/ 9/46	i.p.	0.30	4,669	1.33	1 hour	7	K.	Weakness, dyspn.
	4/11/46	i.m.	0.26	5,861	1.19	1 hour	28	K.	Weakness
	4/11/46	i.m.	0.27	2,504	1.17	1 hour	9	K.	Sl. dyspnea
Dibenzamine hydrochloride	4/23/46	i.m.	0.27	11,104	1.15	24 hours	13	K.	Weakness, gasping
	4/23/46	i.m.	0.25	8,571	1.28	24 hours	13	K.	Weakness, gasping
	4/23/46	i.m.	0.29	11,566	1.05	24 hours	11	S.	Usual features

TABLE 3—*Concluded*

DRUG	DATE	MODE OF INJ. OF EPINE- PHR.	WT. OF HEART (% OF BODY WT.)	AC (COL. UN. PER GM. HEART MUSCLE)	D S R.	TIME FROM DRUG INJ. TO EPINEPHR. INJ.	TIME FROM EPINE- PHR. INJ. TO DEATH	MODE OF DEATH	REMARKS
	4/23/46	i.m.	0.31	12,742	1.29	24 hours	min. 13	K.	Weakness, dyspn.
	4/23/46	i.m.	0.24	16,153	1.41	24 hours	13	S.	Weakness, dyspn.
Dibena- mine hydro- chlo- ride	4/16/46	i.m.	0.31	2,006	1.15	72 hours	6	S.	Usual features
	4/16/46	i.m.	0.34	6,894	1.61	72 hours	11	S.	Usual features
	4/16/46	i.m.	0.31	9,158	1.60	72 hours	6	S.	Usual weakness
	4/16/46	i.m.	0.32	8,691	1.69	72 hours	12	S.	Usual features
(None)	(1941/6)	—	(0.33)*	(1,268)*	(1.11)*	—	—	—	—

* Average of readings obtained in 143 control rats.

† S = spontaneous; K = killed.

TABLE 4
Compilation of average results

TYPE OF EXPERIMENTS	NO. OF ANIMALS	WT. OF HEART (% OF BODY WT.)	AC (COL. UN./GM. HEART M)	D S R.	TIME FROM EPINEPHR. INJ TO DEATH	MORTALITY
					min.	
Untreated controls .	143	0.33	1,268	1.11		%
933F alone ($\frac{1}{2}$ hr.) .	5	0.28	2,111	0.99		
Priscol alone ($\frac{1}{2}$ hr.) .	6	0.31	1,414	1.08		
Dibenamine alone (1 hr.)	5	0.30	2,010	1.08		
Dibenamine alone (24 hrs.)	5	0.33	2,039	1.06		
Epinephrine alone .	10	0.31	5,170	1.35	13	100
933F ($\frac{1}{2}$ hr.) + epin.	10	0.30	2,275	1.44	13	44
Priscol ($\frac{1}{2}$ hr.) + epin.	10	0.29	2,148	1.19	14	10
Dibenamine (1 hr.) + epin.	10	0.28	4,705	1.46	13	0
Dibenamine (24 hrs.) + epin.	5	0.27	12,027	1.24	13	40
Dibenamine (72 hrs.) + epin.	4	0.32	6,687	1.51	9	100

animal died slower than any other (30 min.). The surviving animals showed some transitory weakness and, in one case, dyspnea but no gasping. The average myocardial AC concentration was considerably higher than that found after Priscol alone and well within the fatal range but lower than that found after epinephrine alone. The average d.s.r. was not much elevated (1.19). The

highest tolerated and the lowest fatal AC concentration were not very far above the critical level of 1900 col.un./gm (table 6).

Dibenamine hydrochloride permitted survival of all rats injected with epinephrine within one hour after its administration. The animals showed only epinephrine within one hour after its administration. The animals showed only some transitory weakness and occasional dyspnea, no gasping. The average myocardial AC concentration was about as high as after epinephrine alone, with a correspondingly elevated d.s.r. of 1.46. Even an AC concentration as high as 9.325 was tolerated without major discomfort (table 6). Twenty-four hours

TABLE 5

Epinephrine accumulation in the heart muscle in proportion to dosage (partly compiled from older experiments of the author) and mortality

EPINEPHRINE INJECTED PER GM. BODY WT.	NO. OF EXPERIMENTS	AVERAGE HEART AC OF SUCCEDED RATS	AVERAGE D.S.R.	MORTALITY PER GROUP	MINUTES OF SURVIVAL (AV.)
γ				%	
2.5	15	1,943	1.08	13	12
5.0	15	2,833	1.26	73	8
7.5	6	4,780	1.24	83	5
10.0	10	5,170	1.35	100	13

TABLE 6

Survival of normally fatal myocardial AC concentrations (above 1900 col.un./gm. heart muscle) due to adrenolytic drugs

TYPE OF EXPERIMENTS	NO. OF RATS WITH AC ABOVE 1900 COL.UN./ GM. PER GROUP	AVERAGE OF THESE NORMALLY FATAL VALUES	NO. OF RATS SURVIVING THESE "FATAL" CONC.	HIGHEST AC TOLERATED IN GROUP	LOWEST AC WITH FATAL EFFECT IN GROUP
Epinephrine alone...	10 (100%)	5,170	0 (0%)		2,267
933F ($\frac{1}{4}$ hr.) + epin..	6 (60%)	2,832	2 (33%)	2,134	2,943
Priscol ($\frac{1}{4}$ hr.) + epin.	6 (60%)	2,413	5 (83%)	2,903	2,479
Dibenamine (1 hr.) + epin. ..	8 (80%)	4,705	8 (100%)	9,325	
Dibenamine (24 hrs.) + epin.	5 (100%)	12,027	3 (60%)	12,742	11,566

after administration of Dibenamine hydrochloride the injection of epinephrine was followed by enormous myocardial AC accumulations, yet only 40% of these animals succumbed and the surviving ones were found to have withstood extreme myocardial AC concentrations, reaching as high as 12,742, although with some dyspnea. Seventy-two hours after injection of Dibenamine hydrochloride its protective efficacy had entirely disappeared and the epinephrine injections gave the same results as in untreated animals: 100% mortality and about the same average heart AC.

DISCUSSION. Of the three adrenolytic drugs tested for their efficacy in protecting the heart of the rat against the acutely fatal effect of toxic doses of epineph-

rine, Dibenamine hydrochloride proved to be the most potent. One hour after administration it completely abolished the normally absolutely fatal effect of 10 γ /gm. of epinephrine; twenty-four hours later the protective effect was still present in 60% of the animals; seventy-two hours after administration it had completely vanished. This confirms in part Nickerson's (14) statement of a relatively prolonged action of Dibenamine hydrochloride.

Both Priscol and 933F were also found effective but to a considerably lesser degree than Dibenamine hydrochloride, 933F being the weakest of the three.

As far as the mode of action of these drugs is concerned, it seems improbable that it consists of a chemical destruction of the epinephrine molecule. The chromogenic properties and adsorbability by $\text{Al}(\text{OH})_3$ of epinephrine were not demonstrably altered by exposure to the drugs *in vitro*. Observations by Morison and Lissák (16) regarding a destruction of epinephrine by 933F *in vitro* showed this process to develop so slowly (over many hours) that it can hardly be compared to the immediate effect *in vivo*. Also the fact that injected epinephrine maintains some of its specific non-cardiovascular effects despite 933F (Morin (17)), Priscol (Chess and Yonkman (18)) or Dibenamine hydrochloride (Nickerson (14)) speaks against its chemical alteration through these drugs.

Both 933F and Priscol distinctly inhibited the usual deposition and accumulation of injected epinephrine within the heart muscle, which seems to support Rosenbluth's and Cannon's (19) theory of an impairment of cellular permeability for epinephrine, as far as 933F is concerned, and suggests a similar mechanism for Priscol. The protective effect of both drugs against the moderate amounts of epinephrine which did penetrate into the myocardium was rather limited. However, there seems to be a difference between the actions of these two drugs in that Priscol *per se* did not influence the basic normal concentration of sympathomimetic catechols in the heart muscle while 933F *per se* augmented it. This increase through 933F is not incompatible with the conception of a blocking of the influx of epinephrine from the blood since it can be interpreted as a tendency toward accumulating derivatives of intracellularly discharged sympathin from intracellular sympathetic nerve terminals. Indeed, the behavior of the d.s.r. indicated the non-identity with epinephrine of the material appearing in the myocardium under the influence of 933F alone. The fact that normally fatal AC amounts could accumulate in the heart after injection of 933F without any ill effects suggests the presence also of a certain "sympatholytic" (sympathin-counteracting) action of 933F beside the "adrenolytic" one in agreement with the observations of van Dongen (7) and Martin and Lissák (8).

While Dibenamine hydrochloride did not modify the deposition of injected epinephrine in the heart muscle one hour after its administration, it markedly facilitated this process 24 hours later. Beside, it seemed to favor the influx of spontaneously secreted epinephrine and sympathin into the heart. However, its outstanding feature was its spectacular capacity to render even enormous myocardial epinephrine concentrations innocuous with most of this effect still remaining present 24 hours after administration.

SUMMARY

Three adrenolytic drugs: Dibenamine hydrochloride, Priscol and Fournau's 933F, were found to protect the heart of the rat either completely or partially against normally fatal doses of epinephrine and to permit the survival of normally fatal accumulations of epinephrine in the myocardium.

Dibenamine hydrochloride is by far the most potent, 933F the weakest of these drugs.

Their modes of action are different. Both 933F and Priscol seem to partially block the penetration of epinephrine into the myocardial cells, although not to the same extent; Dibenamine hydrochloride seems to render the heart itself insensitive to epinephrine without impairing, indeed even facilitating its excessive deposition and accumulation in the heart muscle.

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PHARMACOLOGY AND CHEMISTRY OF SUBSTANCES WITH CARDIAC ACTIVITY

IV. EFFECT OF METHYLAMINOETHANOL, DIMETHYLAMINOETHANOL, AND RELATED SUBSTANCES ON THE ISOLATED MAMMALIAN HEART¹

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The erythrophleum alkaloids are esters of tricyclic, possibly diterpenoid acids with β -methylaminoethanol or β -dimethylaminoethanol. They exhibit a characteristic positive inotropic action upon the isolated mammalian heart. Erythrophleic acid, the nitrogen-free product of hydrolysis of the alkaloid erythrophleine, has no cardiac action in doses more than 100 times greater than active amounts of its methylaminoethanol ester (1). Methylaminoethanol and dimethylaminoethanol as well as a number of related alkamines, alkanolamines, and synthetic esters of dimethylaminoethanol have therefore been examined, in order to determine whether or not action on the heart is a property of this group of substances.

The literature contains few references to the pharmacological action of methyl- and dimethylaminoethanol (2). The general biological interest in these compounds has been enhanced by the recent observation that they can serve as precursors of choline in certain biological systems (3, 4, 5, 6). Methylaminoethanol (10 mgm. per animal intravenously) in the rabbit (7), and dimethylaminoethanol (3 mgm. per kgm. intravenously) in the cat (8), were without effect on blood pressure. In the dog, however, both substances (10 to 20 mgm. per kgm. intravenously) lowered the arterial pressure (9). In the only reported observation of a cardiac action of methylaminoethanol, Hauschild (10), found that it caused an increase in the amplitude of contraction in the isolated perfused heart of the cat, rat, and guinea pig. He observed similar effects with aminoethanol and its N-ethyl and N-diethyl derivatives.

METHODS. Twenty-six experiments were carried out on the heart-lung preparation of the dog. The procedure employed in recording changes in the activity of the heart has been described in detail by Krayer and Mendez (11). Defibrinated dog blood was used; the initial total blood volume was between 850 and 900 cc. Heart failure was either spontaneous or produced by administering 0.05 to 0.2 grams of sodium pentobarbital. Changes in competence were followed by recording the response to alterations of blood supply according to the method of Krayer (12). Heart rate was counted directly or from electrocardiographic tracings recorded with an ink-writing oscillograph. Coronary sinus outflow was recorded from a Morawitz cannula, and values have not been corrected to total coronary flow.

In addition to methylaminoethanol and dimethylaminoethanol, the compounds studied included aminoethanol, diethylaminoethanol, choline chloride, betaine hydrochloride,

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tetramethylammonium chloride, tetraethylammonium bromide, diethylaminoethyl diphenylacetate ("Trasentin"—Ciba), and the following esters of dimethylaminoethanol: the acetate, tributylacetate,² cinnamate, benzoate, veratrate, and aetiodesoxycholate. The amines and esters were administered as the hydrochlorides, and all doses refer to the salts.

PREPARATION OF ESTERS. *β -Dimethylaminoethyl acetate hydrochloride* was prepared by the addition of a solution of acetyl chloride in ether to a solution of an equivalent quantity of β -dimethylaminoethanol in ether and recrystallization of the precipitated salt from a mixture of absolute ethanol and ether. M.P., 129–130°.

β -Dimethylaminoethyl benzoate hydrochloride was prepared by the addition of a solution of benzoyl chloride in ether to a solution of an equivalent quantity of β -dimethylaminoethanol in ether and recrystallization of the salt from a mixture of absolute ethanol and ether. M.P., 148–149°.

β -Dimethylaminoethyl veratrate hydrochloride was prepared by the addition of a solution of veratryl chloride (prepared from veratric acid with thionyl chloride) in ether to a solution of an equivalent quantity of β -dimethylaminoethanol in ether and recrystallization of the salt from absolute ethanol. M.P., 165–166°.

β -Dimethylaminoethyl cinnamate hydrochloride was prepared by the addition of a solution of cinnamoyl chloride (prepared from cinnamic acid and thionyl chloride) in ether to a solution of an equivalent quantity of β -dimethylaminoethanol in ether and recrystallization of the salt from absolute ethanol. M.P., 132–133°.

β -Dimethylaminoethyl aetiodesoxycholate hydrochloride. To a solution of 35 mgm. of sodium in 10 ml. of absolute ethanol was added 500 mgm. of aetiodesoxycholic acid³ and the mixture concentrated to dryness *in vacuo*. A solution of 3 g. of β -dimethylaminoethyl chloride in 100 ml. of benzene was added to the dry sodium salt and the mixture was heated under reflux for 48 hours. It was then evaporated to dryness *in vacuo*, treated with ether, and the ether solution extracted with water. The ether extract was dried over anhydrous magnesium sulfate and concentrated to an oil. This residual oil was dissolved in dry ether and a solution of hydrogen chloride in ether was added. The solid which formed was collected and recrystallized from ethyl acetate containing a small amount of absolute ethanol. The yield was 300 mgm. M.P., 240–242°.

RESULTS. I. *Methylaminoethanol and dimethylaminoethanol.* In the normal heart methylaminoethanol and dimethylaminoethanol in doses of 100 to 300 mgm. in a total blood volume of approximately 800 cc. caused a slight decrease in right and left atrial pressure and a correspondingly slight increase in systemic output, thus indicating the potential ability of these compounds to improve the work capacity of the heart. This property was clearly shown in the failing heart (fig. 1), irrespective of whether the failure was spontaneous or induced by drugs such as sodium pentobarbital.

In the experiment represented by fig. 1 the state of failure was such that an increase of 50 mm. in the level of the venous reservoir did not increase the systemic output. A concentration of 1:1500 of methylaminoethanol caused, within one minute, an increase in stroke volume from about 3.5 cc. to 9.7 cc. During the next 4 minutes the stroke volume decreased to about 5.7 cc. as a result of (a) the decrease in systemic output to 550 cc., (b) the return of the

² *β -Dimethylaminoethyl tributylacetate hydrochloride* was supplied by Dr. E. Schwenk, Schering Corporation, Bloomfield, New Jersey. *β -Diethylaminoethyl diphenylacetate hydrochloride* was supplied by Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

³The aetiodesoxycholic acid was supplied by Dr. Willard M. Hoehn of George A. Breon and Company, Kansas City 10, Missouri.

coronary sinus outflow to about the same value as before the injection, and (c) the increase in heart rate to 120 beats per minute. That competence was much improved is evident from the response of the venous pressure and cardiac output to the rise of the blood level in the venous reservoir by 50 mm.

The total blood volume in the heart-lung preparation is distributed between the "system" (consisting of arterial resistance, stromuhr, heating device, venous reservoir and connecting tubes) and the heart cavities and the vessels of heart

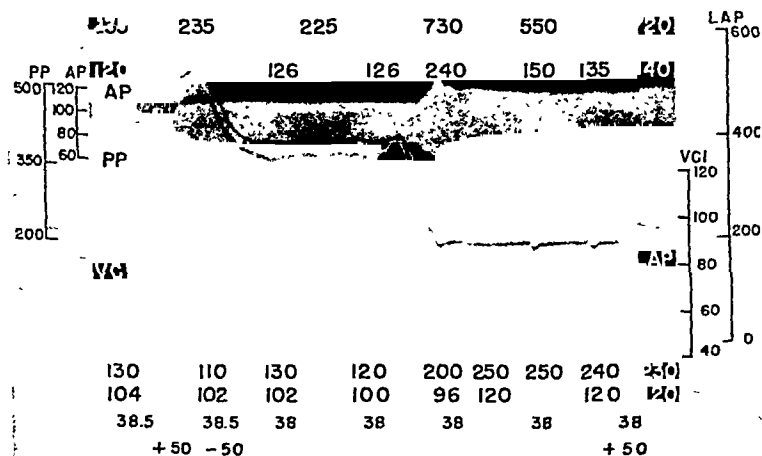


FIG. 1. ACTION OF METHYLAMINOETHANOL HYDROCHLORIDE IN THE FAILING HEART

Heart-lung preparation. Female dog, 10.5 kgm. Anesthesia, sodium pentobarbital, 35 mgm/kgm. Arterial resistance, 71 mm. Hg. Weight of heart ventricles, 88 gram. Cardiac failure produced by 140 mgm. sodium pentobarbital.

Tracings from top to bottom: Systemic output, each signal indicating 100 cc., time in 10 second intervals; arterial pressure (scale on left in mm. of mercury); pulmonary arterial pressure (scale on left in mm. of water); left auricular pressure (scale on right in mm. of water); right auricular pressure recorded from the inferior vena cava (scale on right in mm. of water).

Horizontal rows of figures from top to bottom: Systemic output in cc. per minute; coronary sinus outflow in cc. per minute; blood volume in venous reservoir in cc.; heart-rate per minute; blood temperature in degrees centigrade; at +50 the level of the venous reservoir was raised by 50 mm., at -50 the original level was restored. At signal 400 mgm. of methylaminoethanol hydrochloride was given into the venous reservoir.

and lungs. As the capacity of the "system"—with the exception of the content of the venous reservoir—is rigid, any increase or decrease in the overall capacity of the heart, coronary vessels, and of the vessels of the lungs must lead to a change in the volume of blood in the venous reservoir, provided there is no leak through the vessel walls into extravascular spaces. A change in the amount of blood in the venous reservoir therefore indicates capacity changes in the heart-lung system. After the administration of methylaminoethanol (fig. 1) the

capacity of the vascular bed of the heart and lungs decreased by 130 cc. since the amount of blood in the venous reservoir increased from 120 cc. to 250 cc. Part of this was due to the decrease in diastolic volume of the heart which was apparent on inspection. With a marked drop in pulmonary arterial pressure as in the experiment of fig. 1, it is difficult to say whether the changes in diastolic volume are entirely due to changes in pulmonary resistance (and in heart rate) or, in part at least, to a direct myocardial action. That the latter effect is

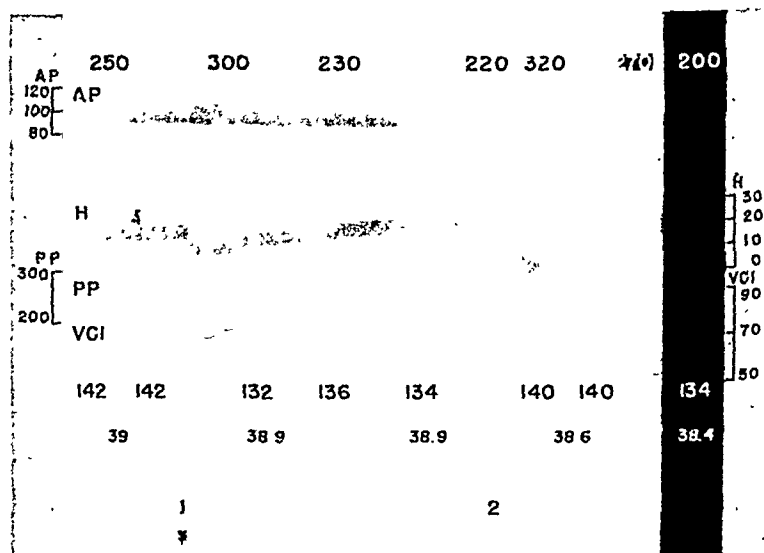


FIG. 2. ACTION OF METHYL- AND DIMETHYLAMINOETHANOL HYDROCHLORIDE ON CARDIAC VOLUME IN SPONTANEOUS FAILURE

Heart-lung preparation. Male dog, 9.4 kgm. Anesthesia, 35 mgm./kgm. sodium pentobarbital intraperitoneally. Arterial resistance, 80 mm. Hg. Weight of heart ventricles, 67 gram. Spontaneous cardiac failure.

Tracings from top to bottom. Systemic output, each signal indicating 100 cc., time in 10 second intervals; arterial pressure (scale on left in mm. of mercury); cardiac volume (scale on right in cc.); pulmonary arterial pressure (scale on left in mm. of water); right auricular pressure recorded from the inferior vena cava (scale on right in mm. water).

Horizontal rows of figures from top to bottom. Systemic output in cc. per minute; heart rate, beats per minute; blood temperature in degrees centigrade. At 1, 200 mgm. dimethylaminoethanol hydrochloride; at 2, 177 mgm. methylaminoethanol hydrochloride. Time interval between first and second portion is 11 minutes.

involved can be more clearly seen from the experiment of fig. 2 on a heart with a slight degree of spontaneous failure, in which a decrease in diastolic volume occurred with little change in pulmonary arterial pressure.

The improvement of the work performance of the failing heart is illustrated by the experiment of table 1, in which the work of the heart has been calculated. A dose of 300 mgm. of dimethylaminoethanol, corresponding to an initial concentration of about 1:2100, improved the competence of the heart and enabled it to carry a work load similar to that of the normal heart.

Heart rate in the isolated heart was ordinarily not changed by either of the two substances (26 experiments); in 6 experiments a slight increase in rate (up to 20% of basal rate) occurred.

Coronary sinus outflow was either not influenced or was slightly decreased when positive inotropic doses were administered to the normal heart. In the failing heart, however, coronary sinus outflow increased regularly and stayed high (table 1), or in some experiments, after a short period, approached the normal value (fig. 1).

Relationship between dose and intensity and duration of action. No quantitative or qualitative differences between methylaminoethanol and dimethylamino-

TABLE 1

Action of dimethylaminoethanol hydrochloride on the heart-lung preparation in sodium pentobarbital failure

Female dog 10.5 kgm. Sodium pentobarbital anesthesia 36 mgm./kgm. Arterial resistance = 70 mm. Hg. Weight of heart ventricles 88 grams. Blood volume = 650 cc.

TIME	SYS- TEMIC OUTPUT	CORO- NARY SINUS OUTFLOW	TOTAL OUTPUT	HEART RATE	STROKE VOLUME	MEAN AORTIC PRESSURE	PULMON- ARY ARTERIAL PRESSURE	RIGHT AURICU- LAR PRESSURE	LEFT AURICU- LAR PRESSURE	WORK OF THE HEART IN KILOGRAM-METERS/MIN.		
										Total	Left heart	Right heart
min.	cc./min	cc./min.	cc./min.	beats/ min.	cc.	mm./Hg	mm. H ₂ O	mm. H ₂ O	mm. H ₂ O			
0	400	20	420	144	2.92	100	200	52	86	0.655	0.571	0.084
7	400	20	420	144	2.92	100	200	52	86	0.655	0.571	0.084
17	410	30	440	146	3.01	100	200	54	88	0.686	0.598	0.088
19	100 mgm. sodium pentobarbital											
35	280	50	330	140	2.35	98	233	75	205	0.517	0.440	0.077
60	200	90	290	124	2.34	95	230	83	281	0.470	0.375	0.096
68	180	90	270	120	2.25	98	371	85	330	0.460	0.360	0.100
70	Dimethylaminoethanol hydrochloride 300 mgm.											
71	400	120	520	118	4.40	102	200	50	123	0.825	0.721	0.104
73	350	120	470	118	3.98	98	200	50	130	0.720	0.626	0.094
82	300	140	440	120	3.66	98	230	75	160	0.658	0.557	0.101
90	275	138	410	118	3.48	98	300	85	225	0.668	0.545	0.123
95	240	130	370	122	3.03	98	320	90	290	0.610	0.492	0.118
100	170	130	300	118	2.54	95	340	110	360	0.490	0.388	0.102

ethanol were observed. The smallest initial dose producing a positive inotropic effect was 50 to 100 mgm. or, in a blood volume of 800 cc., a concentration of 0.5 to 1×10^{-3} molar. It was repeatedly observed that the second dose was more effective than the first, a phenomenon also encountered with the veratrum alkaloids and well illustrated by fig. 3. Subsequent administrations of the same dose did not show a further increase in intensity and duration of action. Maximum positive inotropic effects in failing hearts were observed with doses of 300 to 400 mgm. ($2.3-4 \times 10^{-3}$ M). The duration of action of this dose was of the order of 30 minutes.

Irregularities of the heart beat occurred with large doses and were characterized by auriculo-ventricular dissociation, complete A-V block, idioventricular rhythm,

and ventricular extrasystoles. These toxic changes were studied in 5 experiments with dimethylaminoethanol. Amounts between 0.8 and 1.5 grams ($1.1\text{--}2.2 \times 10^{-2}$ M) had to be given before irregularities occurred. The ratio of minimal irregularity dose to minimal positive inotropic dose is approximately 20. This is a value much higher than for any of the veratrum or erythrophleum alkaloids studied under identical conditions (1, 13).

Influence of atropine and nicotine. Doses of 2 mgm. of atropine sulfate leading to concentrations of 1:300,000 to 1:50,000 in 5 heart-lung preparations did not

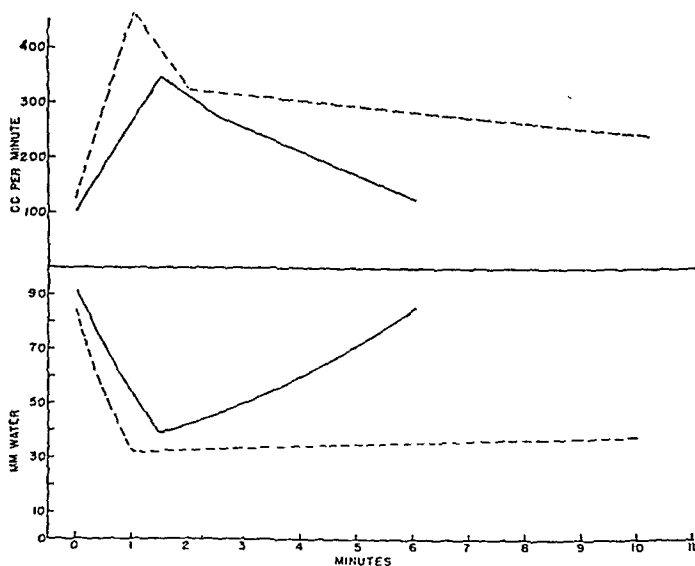


FIG. 3. ACTION OF REPEATED DOSES OF DIMETHYLAMINOETHANOL HYDROCHLORIDE ON THE SYSTEMIC OUTPUT AND RIGHT AURICULAR PRESSURE IN THE HEART-LUNG PREPARATION

Heart-lung preparation. Female dog, 9.7 kgm. Anesthesia, 35 mgm. sodium pentobarbital intraperitoneally; arterial resistance 78 mm. Hg. Weight of heart ventricles, 69.5 gram. Spontaneous failure. Upper section, systemic output; lower section, right auricular pressure. Solid line, first dose of 200 mgm. dimethylaminoethanol hydrochloride; interrupted line, second dose of 200 mgm. dimethylaminoethanol hydrochloride.

significantly modify the positive inotropic action of dimethylaminoethanol. Nicotine in doses of 50 to 80 mgm. (3 experiments) was without influence upon the positive inotropic action of methylaminoethanol and dimethylaminoethanol. These doses of nicotine completely abolished the positive inotropic and positive chronotropic effects of 5 to 10 mgm. of acetylcholine chloride in the atropinized heart (14).

II. Related substances. Several substances related chemically to the two methylaminoethanols were tested for their inotropic and chronotropic action (table 2). Most of the compounds in a dosage range of the same order had a

qualitatively similar effect. Betaine hydrochloride exhibited a negative inotropic effect. Choline chloride showed its positive inotropic action only after atropine had been administered. In the nonatropinized heart it had a negative inotropic, but, in the doses indicated, no negative chronotropic action. Tetramethylammonium ion and tetraethylammonium ion retained their positive inotropic action after nicotine, as did methyl- and dimethylaminoethanol.

Equimolar amounts of aminoethanol were about equal in positive inotropic potency to methylaminoethanol, or dimethylaminoethanol; diethylaminoethanol was somewhat less potent; choline (after atropine) and tetramethylammonium

TABLE 2
Cardiac action of aminoethanol and related compounds

NAME OF COMPOUND	FORMULA	DOSAGE TESTED	BEFORE ATROPINE		AFTER ATROPINE		AFTER ATROPINE + NICOTINE	
			Ino-tropic action	Chrono-tropic action	Ino-tropic action	Chrono-tropic action	Ino-tropic action	Chrono-tropic action
Aminoethanol hydrochloride	$\text{H}_2\text{NCH}_2\text{CH}_2\text{OH} \cdot \text{HCl}$	mgm./l. 155-350	Pos	None	Pos.	None		
β -methylaminoethanol hydrochloride	$\begin{array}{c} \text{CH}_3 \\ \text{H} \end{array} \text{NCH}_2\text{CH}_2\text{OH} \cdot \text{HCl}$	60-800	Pos.	None or slight increase	Pos	None or slight increase	Pos.	None
β -dimethylaminoethanol hydrochloride	$\begin{array}{c} \text{CH}_3 \\ \text{CH}_3 \end{array} \text{NCH}_2\text{CH}_2\text{OH} \cdot \text{HCl}$	15-600	Pos.	None or slight increase	Pos.	None or slight increase	Pos.	None
β -diethylaminoethanol hydrochloride	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \text{C}_2\text{H}_5 \end{array} \text{NCH}_2\text{CH}_2\text{OH} \cdot \text{HCl}$	150-300	Pos.	None	Pos.	None		
Choline chloride	$\{(\text{CH}_2)_2\text{NCH}_2\text{CH}_2\text{OH}\}^+\text{Cl}^-$	70-300	Neg.	None	Pos.	None		
Betaine hydrochloride	$\{(\text{CH}_2)_2\text{NCH}_2\text{COOH}\}^+\text{Cl}^-$	150-300	Neg.	None	Neg.	None		
Tetramethylammonium bromide	$\{(\text{CH}_3)_4\text{N}\}^+\text{Br}^-$	75-350			Pos.	None	Pos.	None
Tetraethylammonium bromide	$\{(\text{C}_2\text{H}_5)_4\text{N}\}^+\text{Br}^-$	15-120	Pos.	None	Pos.	None	Pos.	None

ion were distinctly weaker, while tetraethylammonium ion was more effective than methylaminoethanol.

III. *Esters of dimethylaminoethanol (table 3).* Esterification of dimethylaminoethanol with acetic acid led to the appearance of a marked muscarinic action on the heart (fig. 4). This ester therefore resembles acetylcholine, as was recognized by earlier investigators using other biological systems (15, 16). The benzoate in equimolar amounts exhibited a similar but weaker negative chronotropic action than the acetate. None of the other esters showed muscarinic activity. In no case was the positive inotropic action of dimethyl-

TABLE 3
Cardiac action of dimethylaminoethyl esters

NAME OF COMPOUND	FORMULA	DOSAGE USED	BEFORE ATROPINE		AFTER ATROPINE	
			Ino-tropic action	Chrono-tropic action	Ino-tropic action	Chrono-tropic action
β -dimethylaminoethyl acetate hydrochloride		mgm./l. 28 -220	Neg.	Neg.	Pos.	None
β -dimethylaminoethyl tributyl acetate hydrochloride		5.5- 11.0	Neg.	None	Neg.	None
β -dimethylaminoethyl benzoate hydrochloride		28 -220	Neg.	None	Pos.	None
β -dimethylaminoethyl cinnamate hydrochloride		5.5-110	Neg.	None	Neg.	None
β -dimethylaminoethyl veratrate hydrochloride		5.5-150	Neg.	None	Neg.	None
"Trasentin"		5.5-15	Neg.	None		
β -dimethylaminoethyl aetio-desoxy-cholate hydrochloride		4.2-8.3	Neg.	None		

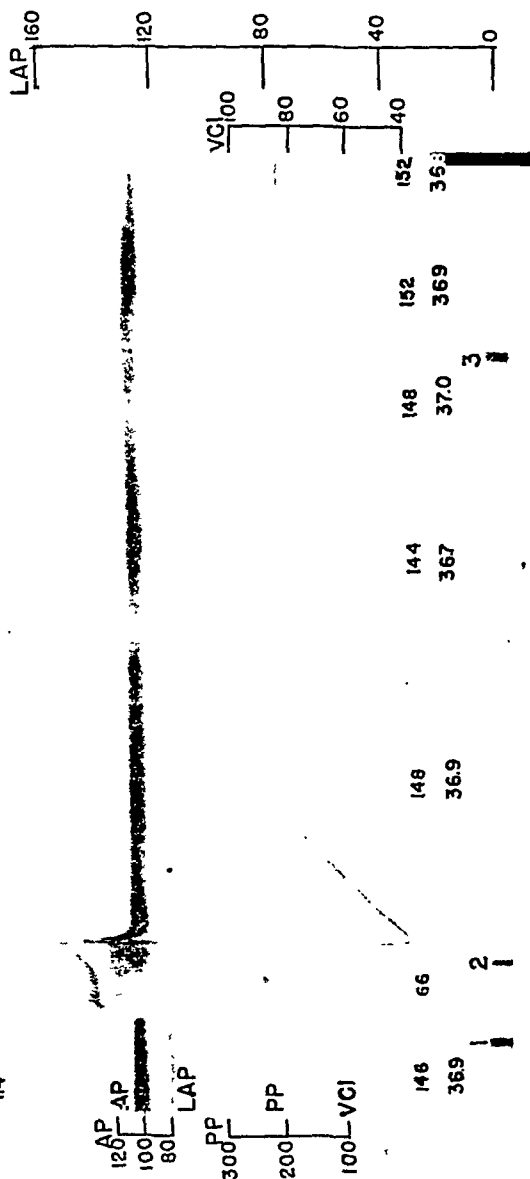


FIG. 4. ACTION OF DIMETHYLAMINOETHYL ACETATE HYDROCHLORIDE ON THE ISOLATED FAILING HEART

Heart lung preparation. Female dog, 9.5 kgm. Anesthesia, 35 mgm./kgm. sodium pentobarbital intraperitoneally. Arterial resistance, 76 mm. Hg. Heart ventricular weight, 81.7 gram.

Tracings from top to bottom: Systemic output, each signal indicating 100 cc., time in 30 second intervals; arterial pressure (scale on left in mm. Hg.); left auricular pressure (scale on right in mm. water); pulmonary arterial pressure (scale on left in mm. water); right auricular pressure recorded from the inferior vena cava (scale on right in mm. of water).

Horizontal rows of figures from top to bottom: Systemic output in cc. per minute; coronary sinus outflow in cc. per minute; heart rate per minute; blood temperature in degrees centigrade. At 1, 200 mgm. dimethylaminoethyl acetate hydrochloride. At 2, 2 mgm. atropine sulfate. At 3, 200 mgm. dimethylaminoethyl acetate hydrochloride.

aminoethanol enhanced by esterification. After atropinization, dimethylaminoethyl acetate was, if anything, less potent than an equimolar amount of the alkanolamine.

DISCUSSION AND SUMMARY

β -Methyl- and β -dimethylaminoethanol have been found to improve the work capacity of the failing isolated mammalian heart in a manner similar to that exhibited by the digitalis glycosides and the epinephrine series. This action is not accompanied by a significant increase in heart rate as is observed with epinephrine, although the effect appears without delay in contrast to the latency period observed with the cardiac glycosides. In addition, the action is not influenced by atropine or nicotine, as opposed to the positive inotropic action of acetylcholine, which appears only after atropine and is inhibited by high concentrations of nicotine (14). The cardiac effects of these amines resemble those elicited by tetraethylammonium bromide (17).

On a molar basis, the simple alkanolamines are approximately two to three times less potent than tetraethylammonium bromide and about 800 times less potent than the erythrophleum alkaloids. With high concentrations, irregularities of heart beat occur, as with all other cardiotoxic drugs studied to the present. The ratio of minimal irregularity concentration to minimal positive inotropic concentration is high. Certain simple substances related chemically to dimethylaminoethanol (table 2) show a qualitatively similar action on the failing heart. A number of esters of dimethylaminoethanol have been prepared and some of them have been found to produce a positive inotropic effect after atropine. In no ester was the potency greater than that of the parent alkanolamine.

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THE ROLE OF HEPATIC DETOXIFICATION IN p-AMINOPROPIOPHENONE INDUCED METHEMOGLOBINEMIA

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During the course of a study of the analgesic properties of various aromatic amines, Vandenbelt and his colleagues (1) discovered that comparatively small doses of p-aminopropiophenone (PAPP) induced the formation of large amounts of methemoglobin in several species without producing any apparent physiological effects other than those attributable to the methemoglobinemia. Jandorf and Bodansky (2) demonstrated the feasibility of protecting dogs against cyanide poisoning by pre-treating them with PAPP. Since the possibility of the prophylactic use of PAPP against cyanide poisoning in man was considered, a study of the relation between the metabolism of the drug and the induced methemoglobinemia was indicated.

MATERIALS AND METHODS. Approximately 150 adult female rats were studied. Their weights varied from 125 to 275 grams, but in most experiments the maximum weight range was ± 10 per cent of the mean. The human experimental subjects included in this study served as subjects in experiments which were reported in detail elsewhere (3).

Methemoglobin was estimated as per cent of total pigment by a slight modification of the Evelyn and Malloy method (4). For early experiments approximately 0.6 cc. of heparinized whole blood was added to 24 cc. of 0.005 *M* phosphate buffer, pH 7.4, and laked with saponin. The absorption was read at 6300 Å. in the Coleman spectrophotometer, as described by Evelyn and Malloy. Since reduction of methemoglobin to hemoglobin was found to occur when the blood was not laked, it was found most convenient to incorporate the saponin in the phosphate buffer as a 0.05 per cent solution. The adaptation of the Evelyn-Malloy method for use with 3 cc. cuvettes in the Beckman Quartz Spectrophotometer permitted the experimenters to secure as many as four serial methemoglobin determinations on the same rat without withdrawing more than 0.5 cc. of blood.

Concentrations of p-aminopropiophenone (PAPP) were determined in blood and urine by a modification of the Bratton and Marshall test for sulfonamides (5). This test depends upon the diazotisation of an aromatic amine with nitrous acid and subsequent coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride to form a pink colored compound which can be compared with appropriate standards in a Klett-Summerson photoelectric colorimeter (filter 54). It must be recognized, therefore, that p-aminopropiophenone as well as any metabolic modification of it which contains a reactive aromatic amino group would give a positive test. *In the present paper any reference to an analytical determination of PAPP is meant to designate the sum of the concentrations of unchanged PAPP and of any transformation products which contain reactive aromatic amino groups.*

Smith (6) has stated that the trichloroacetic acid-filtrate recommended for sulfanilamide

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by Bratton and Marshall is unreliable for p-aminohippuric acid and, possibly, for other aromatic compounds as well. Since PAPP may well have been one of these, experiments were made in which recoveries from solutions of appropriate trichloroacetic acid and saponin concentrations were compared with those from ordinary aqueous solutions and from whole blood. There was no evidence that the use of trichloroacetic acid interfered with the determination.

Experimental procedures. Evisceration was performed by a technique previously described (see Peterson (7) for many references). Rats were anesthetized with sodium pentobarbital (5 mgm. per 100 grams) and a long mid-rectus incision was made. In order, three ligatures were applied to a) the inferior mesenteric artery and rectum, b) the coeliac axis and the middle mesenteric artery, and c) the portal vein, hepatic artery and bile duct. The esophagus, cut at the proximal end of the stomach, was allowed to remain unligated so that swallowing was not inhibited. The spleen, stomach, pancreas and the entire length of the gastrointestinal tract were then removed and the abdominal incision was repaired. Since the duration of the experiments was never more than one hour, supportive glucose therapy was deemed unnecessary.

Rats were bled from the tip of the tail or from the heart, depending on the quantity of blood required. In most cases the blood was heparinized but in those experiments in which approximately 0.10 cc. of blood was used for methemoglobin estimation the blood was allowed to fall directly into the saponin-phosphate buffer solution.

Other details of procedure will be described in connection with the individual experiments to be reported.

RESULTS. Attempts to induce the formation of methemoglobin by PAPP *in vitro* were quite unsuccessful; whole blood incubated for one hour at 37.5°C. showed no accumulation of methemoglobin even when 0.2 mgm. of PAPP per cc. was present in the reaction vessel. This result suggested that methemoglobin is formed in the body not by PAPP, but by a transformation product of the compound. Since the liver was regarded as a probable site for the transformation of PAPP into its methemoglobin forming derivative, the effect of exclusion of the liver from the circulation upon the induction of methemoglobinemia *in vivo* was investigated.

Evisceration experiments. Rats were anesthetized, eviscerated in the manner described above and injected subcutaneously with 5 mgm. of PAPP per kgm. of intact body weight. The compound was given as a 1 per cent solution in propylene glycol. Intact rats were similarly anesthetized and injected with the same dose of the drug. Blood samples drawn 30 and 60 minutes after injection were analyzed for methemoglobin.

Figure 1 shows that at a dose of 5 mgm. of PAPP per kgm. the eviscerate preparations exhibited a *higher* percentage of methemoglobin than did the intact controls; namely, 64.3 per cent versus 18.5 per cent at the end of one hour. Since the dose of the drug was calculated on the basis of intact body weight, the eviscerate preparations received somewhat more PAPP per unit of circulating hemoglobin than did the controls, inasmuch as some blood remained in the gastrointestinal tract which was removed, and some was pooled in the non-functioning liver. However, the magnitude of the difference is such that there can be little doubt that more methemoglobin is formed in the eviscerate preparation than in the control.

That the higher degree of methemoglobinemia attained in the eviscerate animals is associated with a higher concentration of free PAPP (i.e., unconjugated

aromatic amine) is shown in fig. 2. This represents a typical experiment in which the dose of PAPP was 10 mgm. per kgm., injected subcutaneously, and the concentrations of methemoglobin and of PAPP were determined one hour after injection. It is apparent that there is a correlation between the concentration of the drug and the extent of methemoglobinemia, and that the eviscerate animals and the controls fall into two rather distinct groups.

The question arose whether the higher concentration of free PAPP in the blood of eviscerate rats was an absolute rise or was due to a failure of conjugation.

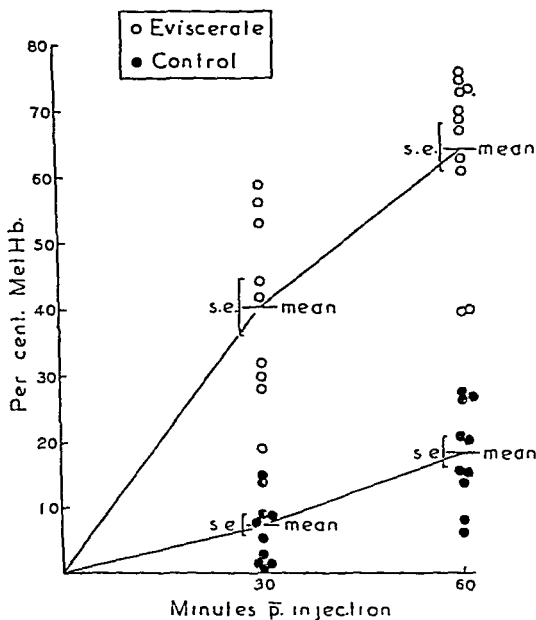


FIG. 1. METHEMOGLOBINEMIC RESPONSE OF INTACT AND EVISCERATE RATS TO p-AMINOPROPIOPHENONE (PAPP)

Dose, 5 mgm./kgm., in 0.1 cc. of propylene glycol, subcutaneously. All rats were injected with sodium pentobarbital dose, 5 mgm./100 grams 15 minutes before injection. Evisceration was performed immediately before injection.

Accordingly, the total concentration of blood PAPP was determined 1 hour after injection of the drug by hydrolyzing aliquots of blood filtrates for 90 minutes at 100°C. in 0.2 N HCl, and these concentrations of total PAPP compared with the concentrations of free PAPP. Figure 3 shows that the higher concentration of free PAPP in the eviscerate rats was due essentially to a failure of conjugation. Thus, in the eviscerate rats 20 per cent of the total PAPP is in the conjugated form, as contrasted with 62 per cent in the intact animals. The concentration of total PAPP was 25 per cent higher in the eviscerate rats than in the controls, but this difference (0.195 mgm. per 100 cc. of blood) may be related to the fact that

(since the dose was calculated on the basis of intact body weight) the compound was dissolved in a smaller volume of fluid in the eviscerate preparations than it was in the controls.

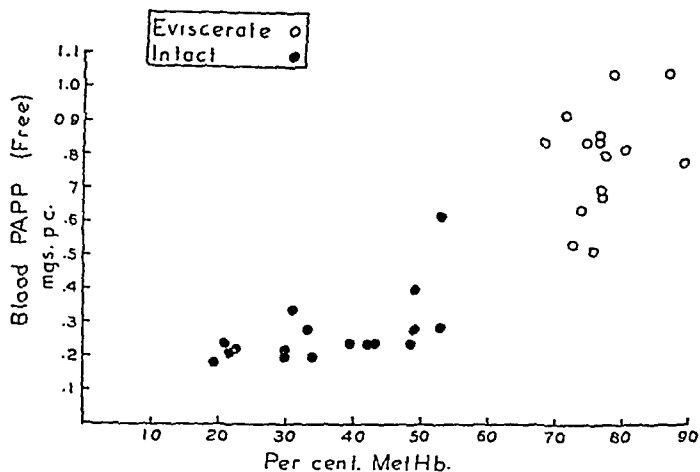


FIG. 2. FREE PAPP IN BLOOD AND METHEMOGLOBINEMIA IN INTACT AND EVISCERATE RATS

Dose, 10 mgm./kgm. in 0.1 ml. of propylene glycol injected subcutaneously. All rats were anesthetized. Concentrations of free PAPP and methemoglobin were determined 60 minutes after injection.

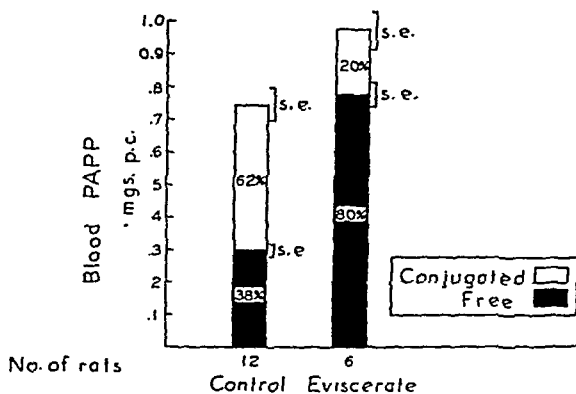


FIG. 3. CONJUGATION OF PAPP IN INTACT AND EVISCERATE RATS

Dose, 10 mgm./kgm., injected subcutaneously. All rats were anesthetized. Concentrations of PAPP were determined 60 minutes after injection.

Conjugation of PAPP by rat liver slices in vitro. The preceding experiments showed a comparative failure of conjugation of PAPP in functionally liverless preparations. Hence, an experiment was designed for the purpose of revealing

whether or not conjugation of PAPP by surviving liver slices could be demonstrated. Duplicate vessels containing 14.8 micrograms of PAPP dissolved in 4 cc. of Hastings' medium (8) were prepared, and to each approximately 200 mgm. of freshly sliced rat liver tissue were added. The flasks were filled with 100 per cent oxygen and shaken in a water bath at 37.5°C. at a rate of 100 oscillations per minute. After incubation and shaking for one hour the slices were ground with a stirring rod, protein was precipitated with a final concentration of 4 per cent. Trichloroacetic acid, and free and conjugated PAPP were estimated on 1.0 cc. aliquots of the protein-free filtrate. The medium with dissolved PAPP and the same solution plus liver slices killed immediately with trichloroacetic acid were used as controls. The results of this experiment are given in fig. 4; they show conclusively that the liver slices conjugated from 64 to 86 per cent of the PAPP recovered at the end of one hour. They show, too, that only about seventy per

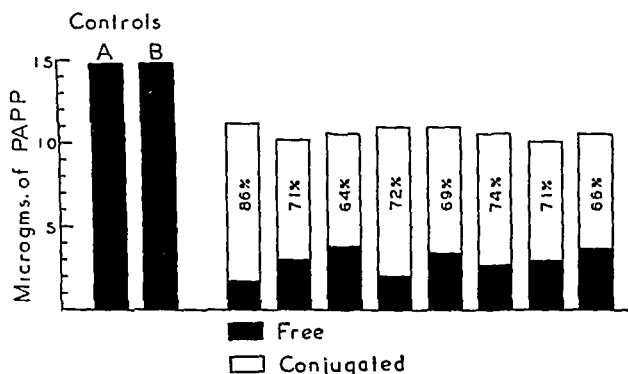


FIG. 4. CONJUGATION OF PAPP BY SURVIVING LIVER SLICES

A: medium containing 14.8 micrograms of PAPP per 4 cc.

B: same medium plus 200 mgm. of liver slices immediately treated with trichloroacetic acid.

All other flasks contained approximately 200 mgm. of liver slices, 4 cc. of PAPP containing medium and oxygen. Determinations were made at end of 1 hour.

cent of the PAPP was recovered on hydrolysis under the conditions of this experiment. This failure to recover the compound quantitatively suggests that, in addition to conjugating the aromatic amino group of PAPP, the liver slices may also oxidize the compound at this group. This process has been shown by Bernheim and Bernheim (9) to occur with similar compounds in the presence of liver slices.

Urinary Excretion of PAPP. The experiments described above show that PAPP is conjugated in the rat, and that this conjugation can be demonstrated in the presence of surviving rat liver slices. It was pertinent, then, to inquire whether or not a similar conjugation occurs in man. Therefore, 1.25 mgm. of PAPP per kgm. of body weight, dissolved in 10 cc. of propylene glycol, was ingested on seven occasions by five adult male human subjects, and free and total PAPP analyses were made of their urine collected for 24 hours after the drug was

administered. As table 1 shows, between 65 and 90 per cent of the administered dose was accounted for in the urine, and of that, approximately eighty per cent was excreted as a conjugated compound.

Distribution of free and conjugated PAPP in red blood cells and plasma. Since a positive correlation was found to exist between the concentration of free PAPP in blood and the extent of methemoglobinemia, it was pertinent to inquire

TABLE 1
Excretion of PAPP by human subjects

SUBJECT	PER CENT OF TOTAL DOSE EXCRETED IN 24 HOURS	PER CENT CONJUGATED
M	73	80
D	76	80
D	71	79
O	83	83
H	65	81
S	90	77
S	86	76

Dose of PAPP, 1.25 mgm./kgm. in propylene glycol

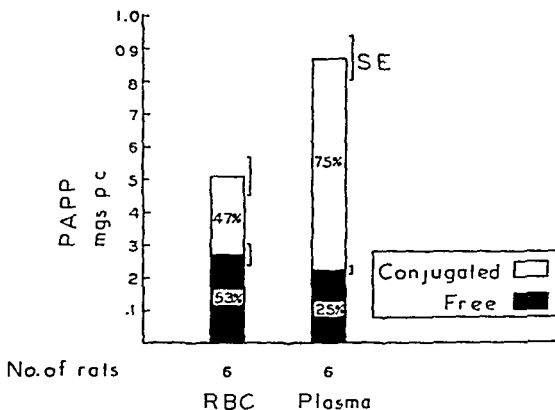


FIG. 5. DISTRIBUTION OF PAPP IN CELLS AND PLASMA OF RATS

10 mgm./kgm. injected into unanesthetized rats (i.p.). Determinations were made 60 minutes after injection.

whether or not the aromatic amino group of the compound was modified within the red cell, in the presence of hemoglobin. It was also of interest to determine the distribution of the conjugated compound in red blood cells and plasma. Therefore, six rats were injected intraperitoneally with 10 mgm. of PAPP per kgm. of body weight, and PAPP analyses were performed on whole heparinized blood and plasma 60 minutes after injection. Since hematocrits were done on each blood sample, it was possible to calculate the concentrations of PAPP in the

red blood cells. Inspection of fig. 5 reveals that whereas there is no statistically significant difference in the concentration of free PAPP in cells and plasma, there is a decided difference in the distribution of conjugated PAPP; i.e., there is only about one-third as much in the red cells as in the plasma. It was concluded from this experiment that the estimation of free PAPP in whole blood constitutes an accurate index of the amount of free PAPP in the red blood cells, and that the red blood cell membrane is not as freely permeable to the conjugated compound as it is to the free form.

Since it is well known that such compounds as p-aminobenzoic acid and many sulfonamides are acetylated in the liver, the possibility that PAPP is similarly converted was considered. Synthetic acetyl-PAPP was injected subcutaneously in an amount equivalent to 10 mgm. of PAPP per kgm. of body weight, and blood was tested for the presence of methemoglobin, and for free and conjugated PAPP. No free PAPP or methemoglobinemia was detected in the blood of animals so treated. However, the distribution of acetyl-PAPP in red blood cells and plasma closely approximated the distribution pattern of PAPP conjugated *in vivo*.

Potential of the methemoglobin-forming action of PAPP. Since it was inferred from the preceding experiments that the conjugated form of PAPP probably is not active in the formation of methemoglobin, it was postulated that the rate of conjugation of the compound has a marked effect on the methemoglobinemia resulting from the injection of a given dose of the drug. Further, if the rate of conjugation of PAPP could be altered, it was believed that this change would be reflected in the degree of methemoglobinemia produced by the compound. The following experiments were devised to test this hypothesis. Since sodium sulfadiazine contains an aromatic amino group and therefore might be expected to be conjugated by a mechanism related to that involved in the conjugation of PAPP, it was postulated that this sulfonamide would compete successfully with PAPP for the available conjugating mechanism and therefore would allow a given dose of PAPP to produce more methemoglobin than might otherwise occur. It was found that sodium sulfadiazine itself produced no detectable methemoglobinemia in the dose employed. Therefore, six animals were given a very large dose (2.5 gram per kgm. of body weight) of sodium sulfadiazine by stomach tube, and six controls were given an equivalent amount of saline, approximately 5 cc. One hour following the tube-feeding each animal was injected intraperitoneally with 10 mgm. of PAPP per kgm. of body weight and blood samples were taken for methemoglobin analysis one, two and three hours after the injection. The results of this experiment are shown graphically in fig. 6. Clearly, the sulfadiazine pre-treated rats showed a higher degree of methemoglobinemia than did the controls; three hours after injection the mean value for the former group was 55 per cent, whereas the corresponding value for the controls was 24 per cent. Not only was there an upward displacement of the methemoglobin response curve, but standard errors in the sulfadiazine group were smaller than were those of the control group.

A similar experiment was done with p-aminobenzoic acid (PABA), another

non-methemoglobin-forming aromatic amine. In this experiment the pre-treated animals were given 0.2 gram of PABA (dissolved in propylene glycol) per kgm. of body weight, while the controls were given appropriate amounts of propylene glycol. Again, one hour after tube-feeding, each animal was injected intraperitoneally with 10 mgm. of PAPP per kgm. of body weight and four serial blood samples, taken at one-hour intervals, were analyzed for methemoglobin. The results are plotted in fig. 7. While the maximal methemoglobinemia attained at one hour by the PABA group was similar to that seen in the sulfadiazine experiment, a high level was maintained for an appreciably longer time,

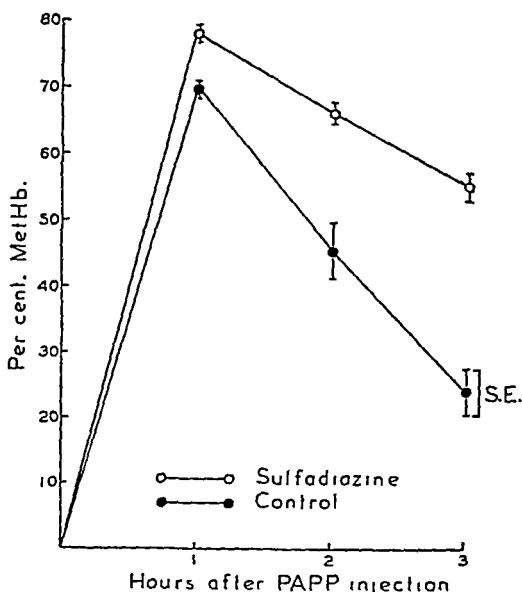


FIG. 6. METHEMOGLOBINEMIC RESPONSE TO PAPP IN SULFADIAZINE PRETREATED RATS AND CONTROLS

Dose of PAPP, 10 mgm./kgm., intraperitoneally. Six unanesthetized animals in each group. Pretreated rats were given 2.5 grams of sodium sulfadiazine per kgm. by stomach tube one hour before the PAPP injection.

so that the difference at 4 hours was very large indeed (60 per cent versus 13 per cent). Again, the diminution of scatter in the PABA group is apparent. This suggests that one of the variables in the response of normal animals to PAPP may be the rate of conjugation of the compound, since treatment which may constitute inhibition of conjugation decreases the variability of the response to the drug.

Development of increased tolerance to PAPP. If inhibition of the conjugating mechanism of PAPP results in a greater and longer sustained rise in methemoglobinemia than is seen in normal animals, an increased efficiency in conjugation might reasonably be expected to bring about a smaller rise following the admin-

istration of a given dose of the drug. During the course of an experiment on the renal excretion of PAPP by the rate in which the compound was injected twice daily for two weeks. the animals appeared clinically to be less affected by a single dose of the drug at the end of the experiment than they had at the beginning. Albaum and Bodansky (10) had measured the methemoglobinemic response to a single orally ingested dose of PAPP in dogs before and after daily administration of the drug for two weeks, and, on the basis of their results, had suggested the possibility that repeated administration of PAPP may be accompanied by "training" effects.

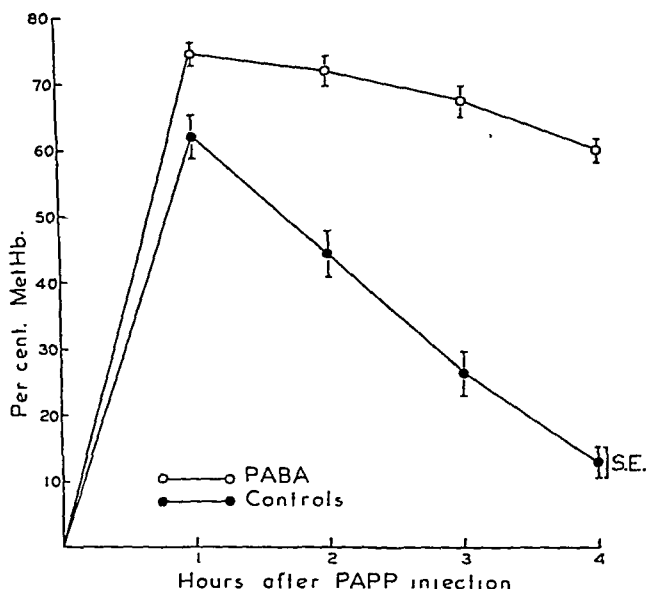


FIG. 7. METHEMOGLOBINEMIC RESPONSE TO PAPP IN PABA PRETREATED RATS AND CONTROLS

Dose of PAPP, 10 mgm./kgm. intraperitoneally. Six unanesthetized rats in each group. Pretreated animals were given 1.0 gram of PABA per kgm. by stomach tube one hour before the PAPP injection.

Experiments were carried out to test this hypothesis. Eight rats were injected intraperitoneally with 5 mgm. of PAPP per kgm. of body weight twice daily, morning and afternoon. Since the drug was given dissolved in propylene glycol, five controls were similarly injected with appropriate amounts of that solvent. (It was established that there was virtually no circulating methemoglobin in the PAPP-injected animals on the morning after the preceding injection, about 18 hours before.) After two weeks on this schedule each animal was given a single intraperitoneal dose of 10 mgm. of PAPP per kgm. of body weight and three samples of blood were drawn at hourly intervals and analyzed for

methemoglobin. A graphic record of this experiment is given in fig. 8. The PAPP "trained" animals uniformly showed a smaller rise in methemoglobin, 46 per cent at one hour as opposed to 61 per cent in the propylene glycol controls.

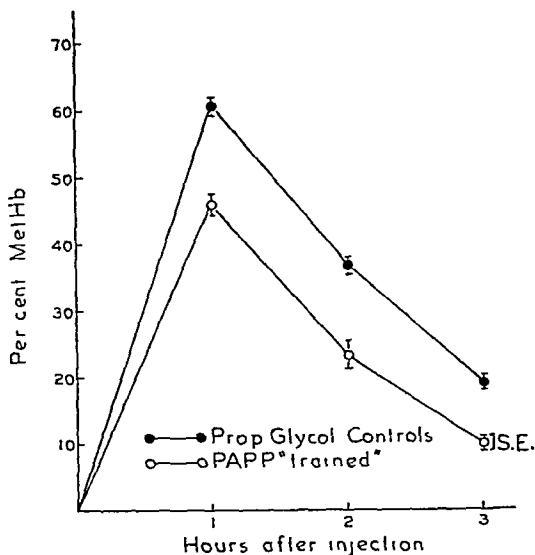


FIG 8 EFFECT OF REPEATED INJECTIONS OF PAPP ON THE METHEMOGLOBINEMIC RESPONSE TO THE DRUG

"Trained" rats were given 5 mgm. of PAPP per kgm. twice daily for two weeks before testing. The test dose of PAPP was 10 mgm./kgm., injected intraperitoneally.

TABLE 2

Formation of methemoglobin *in vitro* by plasma of PAPP-injected rats

RATIO, VOL. DONOR PLASMA* VOL. RECIPIENT CELLS	MET HB FORMED IN 5 MINUTES PER CENT
0:1	0.2
2:1	1.5
4:1	2.3
8:1	4.0
16:1	4.7
32:1	5.3
32:0	0.0

Formation of methemoglobin by a derivative of PAPP in vitro. The foregoing experiments show that one fate of injected or ingested PAPP involves its conjugation by an hepatic mechanism and its subsequent excretion in the urine as a conjugated compound. It is also possible that oxidative deamination of the drug occurs to a small extent. However, since PAPP does not induce the formation of methemoglobin *in vitro*, an alternative metabolic pathway must exist,

a pathway whereby PAPP is converted into the substance which directly oxidizes hemoglobin to methemoglobin.

The following experiment was designed to demonstrate the existence of a derivative of PAPP which is capable of inducing the formation of methemoglobin *in vitro*. Eight rats were injected intraperitoneally with 10 mgm. of PAPP per kgm. of body weight; one hour later each was anesthetized with pentobarbital and exsanguinated from the heart with a heparinized syringe. An uninjected rat was used as a source of red blood cells. The blood of the injected rats was centrifuged and those samples which were visibly hemolyzed were discarded. The plasma of the remaining samples was pooled (donor plasma) and mixed with red blood cells which contained no methemoglobin (recipient cells) in the following ratios of plasma to cells: 2:1, 4:1, 8:1, 16:1, and 32:1. The mixtures were made up to a volume of 3 ml. in each case and shaken at room temperature for about 5 minutes. Samples were then taken from each for methemoglobin estimation. The experimental results are given graphically in table 2. Appreciable quantities of methemoglobin were formed; the maximum amount, 5.3 per cent, was obtained when 32 volumes of donor plasma were mixed in one volume of recipient cells. The plasma itself, even in the highest concentrations employed, did not contain a demonstrable quantity of methemoglobin.

Discussion. Studies on acetanilid have suggested that this compound may be converted to p-aminophenol in the course of its "activation" as a methemoglobin former (see Clark et al. (11) for references). Since p-aminophenol forms methemoglobin readily *in vitro*, the suggestion was made that it may be an intermediary compound in the "activation" of PAPP. That this is improbable is demonstrated by the fact that the dose of p-aminophenol required to produce a methemoglobinemia of about 25 per cent in the rat is approximately ten times as great as the dose of PAPP required to produce a similar response. This is in partial confirmation of the work of Scheff (12).

Calculations were made from data presented as fig. 3 to illustrate the order of magnitude of the molar relationship between free PAPP circulating in the blood and hemoglobin transformed to methemoglobin. These data were obtained on blood samples drawn one hour after the subcutaneous injection of 10 mgm. of PAPP per kgm. of body weight. In these experiments, the average methemoglobin concentration was 75 per cent, and the concentration of PAPP was approximately 0.8 mgm. per 100 cc. of blood. On the basis of these figures, and assuming a concentration of 16 grams of hemoglobin, and a molecular weight of 68,000 for hemoglobin, the molar ratio of methemoglobin iron to PAPP is 132:1. According to the results shown in fig. 2, approximately 35 per cent methemoglobinemia is associated with concentrations of approximately 0.25 mgm. of PAPP per 100 cc. of blood. Calculation from these values yields a ratio of 196:1.

The possibility exists that these high ratios were due to the fact that, at the end of 60 minutes, when the concentrations of methemoglobin and PAPP were determined, a considerable portion of the PAPP which had already reacted with hemoglobin to form methemoglobin had been changed to a form which did not give the Bratton-Marshall test or had been excreted. However, even if it is

assumed that the entire dose, 10 mgm. of PAPP per kgm. body weight (or 1.0 mgm. of PAPP per 100 cc. of blood) takes part in the reaction, the methemoglobin formed is still far in excess (10:1 to 20:1) of the PAPP available for a direct oxidation. These findings strongly suggest, therefore, that PAPP participates in a "turnover" reaction; i.e., that the reduced derivative of PAPP is capable of being regenerated to the oxidized form in the animal body, and thus enabled again to oxidize another portion of hemoglobin iron to methemoglobin. The proof of this hypothesis must await more precise characterization of the postulated "active" intermediary of PAPP. At present nothing is known about the nature of this intermediary compound. As long ago as 1913, Heubner (13) made an extensive study of methemoglobin formation in the presence of derivatives of the p-aminophenols. At that time he suggested that a preliminary oxidation of p-aminophenol to iminoquinone occurred, and that hemoglobin is oxidized to methemoglobin with the re-formation of p-aminophenol. This theory is still widely held (for example, see Brownlee (14)) although the existence of the intermediary quinone has not been proved. One can only suggest the possibility that a similar mechanism may be operative in the case of PAPP.

The methemoglobinemic response to a given dose of PAPP depends on an interrelated series of rates: the rate of absorption of the compound into the bloodstream, the rate of conjugation, and, possibly the rate of oxidation as well, the rate of excretion, the rate of conversion of the active oxidant. In addition to these, the extent and duration of methemoglobinemia resulting from the injection of a single dose of the drug is strongly influenced by the reduction of formed methemoglobin to hemoglobin. Thus the typical methemoglobinemic response curve can be analyzed in terms of the various factors which are simultaneously tending to raise and lower it. During the first half hour not much of the drug has been conjugated, and the methemoglobin-forming reactions gain the ascendancy. By the end of the first hour in the intact rat over 60 per cent of the circulating PAPP has been conjugated. Between the end of the first and second hours there is the beginning of a sharp fall in methemoglobinemia. This suggests that the reduction of methemoglobin to hemoglobin in the red cell (15) has begun to occur at a rate that outstrips methemoglobin formation under the influence of residual (i.e., non-conjugated) PAPP. The response to a given dose can be accentuated by inhibiting the conjugating mechanism or diminished by inducing adaptive "training" of it.

SUMMARY

In the absence of the liver a single dose of p-aminopropiophenone (PAPP) induces the formation of more methemoglobin than it does in an intact animal. This effect was found to be related to the conjugation of the compound by liver tissue. This conjugation occurs in both man and the rat.

There is a positive correlation between extent of methemoglobinemia and the concentration of free PAPP in the blood.

Free PAPP penetrates freely into the red blood cell, but the conjugated form shows a plasma : cell ratio of about 3:1.

Pretreatment of rats with non-methemoglobin forming aromatic amines (sulfadiazine and p-aminobenzoic acid) results in a higher and more prolonged methemoglobinemia than occurs in normal animals injected with the same dose of PAPP. Rats injected twice daily with PAPP in propylene glycol exhibit a smaller methemoglobinemic response from a single dose of PAPP than do control animals.

Altho PAPP does not induce the formation of methemoglobin *in vitro*, plasma obtained from PAPP-treated rats does so to an appreciable extent.

The nature of the reaction between PAPP and hemoglobin is discussed in the light of the findings herein reported.

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METABOLISM OF TRINITROTOLUENE (TNT) IN VITRO¹

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Toxic effects from trinitrotoluene (TNT) have been encountered where this compound has been manufactured and handled on a large scale. In workers engaged in these occupations gastrointestinal disorders, aplastic anemia, polymorphocytosis and toxic jaundice have been observed (1, 2, 3).

For a better understanding of the mechanism of the toxic action of TNT, studies of its metabolism in vitro and in vivo are indicated. In the present study it was found that in the presence of certain tissues, in vitro, TNT undergoes a stepwise reduction of one nitro group with the formation of 4-amino-2,6-dinitrotoluene as an endproduct. In this reduction, an intermediate compound is 4-hydroxylamino-2,6-dinitrotoluene. The reduction of TNT is initiated by flavoprotein-enzymes.

Since this work was completed two papers (4, 5) have appeared describing the isolation and identification of 4-amino-2,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene from the urines of rabbits, rats and men fed or exposed to TNT. Thus, the reduction of TNT in the tissues occurs in vitro as well as in vivo.

EXPERIMENTAL Aqueous stock solutions of TNT were prepared by shaking mechanically trinitrotoluene (Eastman Kodak) (recrystallized twice from alcohol) in water or sodium chloride solution (0.9 per cent). Tissue extracts or enzyme preparations were incubated anaerobically with TNT solutions in Thunberg tubes which were evacuated and then filled with oxygen free nitrogen three times in succession.

Analytical methods The oxygen uptake of tissue slices was measured in a Krebs Ringer solution (6) in the Warburg apparatus at 37°C. The first reading was made after allowing 10 minutes for temperature-equilibration.

TNT was determined colorimetrically by measuring the reddish purple color produced on addition of alcoholic KOH to a solution of TNT in an alcohol ether mixture. Under these conditions solutions of hydroxylamino dinitrotoluene become brownish red, solutions of tetranitroazoytoluene develop a deep blue color, and solutions of amino dinitrotoluene remain colorless. In a mixture containing both tetranitroazoytoluene and TNT, these two products can be determined separately when the decrease in transmission after addition of KOH is recorded with two different filters (#54 and #66 of the Klett Summerson Colorimeter). If an aqueous solution of the hydroxylamino derivative is heated on a boiling water bath for 10 minutes, it is converted into the azoxy compound while TNT remains unchanged. Thus, TNT and the sum of azoxy and hydroxylamino dinitrotoluene can be determined. The azoxy compound is determined separately using a similar procedure.

¹The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

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without previous heating. With filters #54 and #66 the absorption was found to be proportional to the concentration of these compounds.

Since none of azoxy-compound could be detected, in any of the experiments described below, the method described in the following deals only with the determination of a mixture of TNT and hydroxylamino-dinitrotoluene in the biological materials used:

One ml. of the mixture to be analyzed is adjusted to a pH between 6.5 and 6.0 with a predetermined volume of acetic acid (0.05 *N*) and then heated for 10 minutes on the boiling water bath in a tube covered with a glass bulb. After cooling to room temperature the pH is adjusted to approximately 8.0 with a predetermined volume of a solution of K_2PO_4 (0.1 *M*) and 6 ml. of a 1:1 mixture of alcohol-ether³ are added. The mixture is centrifuged and 5 ml. of the supernatant are added to a colorimeter tube containing 0.5 ml. of water. 0.2 ml. of alcoholic KOH (5 per cent), are then added and after mixing thoroughly the color produced is read in a Klett-Summerson colorimeter, using filters #66 and #54.

Calculation: The following factors $\left(\frac{\text{colorimeter reading}}{\text{micrograms}} \right)$ were found:

	Filter #66	Filter #54
Trinitrotoluene	... 2.9	6.4
4-hydroxylamino-2,6-dinitrotoluene	.. 1.9	1.2

Let R_{54} and R_{66} be the colorimeter readings with filters #54 and #66, respectively; T the micrograms of TNT; and H the micrograms of hydroxylamino-dinitrotoluene in the mixture to be determined, then:

$$(1) \quad R_{54} = 6.4 \times T + 1.2 \times H$$

$$(2) \quad R_{66} = 2.9 \times T + 1.9 \times H$$

$$1.9 \times H = R_{66} - 2.9 \times T$$

$$H = \frac{R_{66} - 2.9 \times T}{1.9}$$

$$R_{54} = 6.4 \times T + \frac{1.2 \times (R_{66} - 2.9 \times T)}{1.9}$$

$$R_{54} = 6.4 \times T + 0.63 \times R_{66} - 1.84 \times T$$

$$4.56 \times T = R_{54} - 0.63 \times R_{66}$$

$$T = \frac{R_{54} - 0.63 \times R_{66}}{4.56}$$

and

$$H = \frac{R_{66} - 0.45 \times R_{54}}{1.36}$$

Amino-dinitrotoluene was determined by Westfall's (7) modification of the method of Bratton and Marshall (8) for the determination of sulfanilamide derivatives. It was found that one-fifth of the hydroxylamino-compound present in the mixture is diazotizable. The necessary corrections were made accordingly. Aromatic nitro groups were determined with titanous chloride, according to Kolthoff (9).

Preparations: 4-hydroxylamino-2,6-dinitrotoluene (Mp. 140-141°), 2,2'-6,6'-tetranitro-

³ All ether used for these determinations and for the isolation of the compounds described later was treated with ferrous sulfate and then distilled over KOH.

4-azoxytoluene (Mp. 212-213°), and 2,6-dinitro-4-aminotoluene (Mp. 170-171°) were synthesized according to Anschütz and Zimmermann (10).

Pig heart extracts: After grinding the fresh organ, the tissue (100 gm.) was washed 3 times with ice cold tap water (2000 ml.) by mechanical stirring. After each washing the fluid fraction was squeezed out through a cheese cloth. The muscle mince was then homogenized in a Waring Blendor with 0.01 M Na_2HPO_4 (400 ml.). Freshly prepared calcium phosphate gel (15 ml.) was then added slowly to the homogenate with constant stirring. The mixture was centrifuged, the supernatant discarded and the residue eluted with 0.5 M K_2HPO_4 (200 ml.). The eluate, when stored at +4°C., was stable for at least 2 weeks. In some experiments it was purified by precipitation with ammonium sulfate (15 per cent) and redissolving the residue in K_2HPO_4 (0.02 M) followed by dialysis against the same solution. During all operations particular care was taken to have all solutions and vessels well cooled with cracked ice.

Acetone Extracts of Liver: Fresh pig liver was washed free of blood with a solution of sodium chloride (0.9 per cent). The organ was ground thoroughly and then homogenized with 5 volumes of acetone,⁴ cooled to -10°C. The homogenate was filtered over Buchner

TABLE 1
Inhibition of oxygen uptake of rat liver and muscle slices by TNT

TISSUE	TNT CONCN.	MG. TNT PER GM. OF DRY TISSUE INITIALLY PRESENT	1ST 30 MIN.		2ND 30 MIN.		3RD 30 MIN.		MG. TNT REMOVED PER 1 GM. OF DRY TISSUE	
			O_2	% inhibition	O_2	% inhibition	O_2	% inhibition	0-30 min.	30-90 min.
Liver	mg. %									
	0		9.8		10.0		9.8			
	1.25	2.7	9.6	2	9.3	7	8.5	13	1.0	0.4
	2.5	5.6	9.4	4	3.9	61	3.9	60	3.4	0.9
	5.0	9.8	9.6	2	3.1	69	2.4	75	5.8	1.3
Diaphragm	10.0	20.6	9.4	4	3.1	69	1.6	82	8.6	0
	0		6.0		5.8		5.5			
	1.25	2.2	5.8	3	5.3	9	4.8	13	0.7	0.2
	2.5	5.3	6.0	0	3.2	45	2.4	56	2.4	0.5
	5	8.7	5.9	1	2.4	59	1.9	65	3.8	0.8

funnels, the residue suspended in another 5 volumes of acetone (-10°C.), and filtered again. This operation was repeated once more. The residue was then suspended in a similar volume of peroxide-free ice cold ether, filtered, dried in vacuo and then sifted through a sieve in order to remove connective tissue. The powder obtained in this manner was stable for at least one week when stored in the refrigerator. An aqueous extract was prepared by homogenizing this powder in 10 volumes of ice cold phosphate buffer (0.15 M; pH 7.6) and centrifuging off the residue.

Succinic dehydrogenase was prepared according to Stotz and Hastings (11), diphosphopyridine nucleotide (DPN) according to Ochoa's (12) modification of the method of Green and Williamson (13), reduced DPN according to Warburg, Christian and Griese (14), the flavine enzyme according to Straub (15), and xanthine oxidase (till step 3) according to Corran, et al. (16).

⁴The acetone had been freshly redistilled over anhydrous K_2CO_3 ; the fraction boiling at 56.5°C. was used.

RESULTS. *Effect of TNT on tissue respiration and TNT removal by tissue slices and homogenates.* When either liver slices or diaphragm of rats are shaken with TNT in Krebs-Ringer solution, the TNT disappears rapidly in the first 30 minutes. This removal of TNT is not accompanied by a change in the rate of respiration of the tissues during the early stages of the experiment, although thereafter a decrease in O_2 uptake occurs (table 1). It appears, therefore, that TNT is converted into a compound which inhibits tissue respiration, although TNT itself has no such effect. If, after a period of 90 minutes, the tissue slices

TABLE 2
Effect of metabolized TNT on tissue respiration

SOLUTION	TISSUE	1ST 30 MIN.		2ND 30 MIN.		3RD 30 MIN.	
		QO_2	% inhibition	QO_2	% inhibition	QO_2	% inhibition
Krebs-Ringer solution shaken previously in O_2 with liver slices for 90 min. (no TNT)	Liver	11.1		10.8		10.9	
	Diaphragm	6.2		6.0		6.0	
Krebs-Ringer solution containing TNT shaken previously in O_2 with liver slices for 90 min. Initial TNT: 5.0 mg.%. Final concn: 1.3 mg.% TNT	Liver	11.0	1	10.6	2	11.0	0
	Diaphragm	6.3	0	6.0	0	5.9	2
Krebs-Ringer solution shaken previously in N_2/CO_2 with rat liver slices for 100 min. (no TNT)	Liver	10.3		10.0		10.0	
	Diaphragm	6.1		6.0		5.6	
Krebs-Ringer solution containing TNT shaken previously in N_2/CO_2 with rat liver slices for 100 min. Initial TNT: 5 mg.%. Final TNT concn.: None.	Liver	10.4	0	9.7	3	9.9	1
	Diaphragm	6.2	0	5.9	2	5.7	0
Same solution with added TNT. (Final concn: 5.0 mg.%)	Liver	10.3	0	8.2	18	5.2	48
	Diaphragm	5.9	4	4.7	22	2.5	55

which had been shaken in a TNT solution are removed and replaced by fresh slices, the previously noted inhibitory effect on respiration disappears. The inhibitory compound produced from TNT by muscle and liver slices is apparently further metabolized, with the formation of a substance which has no depressant effect on the respiration of these tissues. As illustrated in table 2, this occurs both after aerobic and anaerobic incubation of liver slices with TNT. Similar observations were made with Krebs-Ringer solutions of TNT shaken aerobically or anaerobically with rat diaphragm. These results indicate that the metabolism of TNT *in vitro* proceeds through at least two steps: first, TNT is converted into a

compound having an inhibitory effect on tissue respiration; and second, from this product a substance is then formed which does not depress the consumption of oxygen.

The removal of TNT by tissue slices proceeds at a much faster rate under

TABLE 3

TNT removal by liver and muscle slices of rats under aerobic and anaerobic conditions

TISSUE	GAS	MG. % TNT		MG. TNT REMOVED IN 10 MIN. PER GRAM DRY TISSUE
		Initial	Final	
Liver	O ₂	10.0	7.6	3.8
	N ₂	10.0	2.3	10.4
Liver	O ₂	10.0	8.2	2.1
	N ₂	10 0	1 1	14.6
Diaphragm	O ₂	5.0	4 1	0.8
	N ₂	5.0	0.7	3.8
Diaphragm	O ₂	5 0	4 5	0.4
	N ₂	5 0	2 6	2.2

TABLE 4

Removal of TNT by homogenized rat tissues

The tissues were homogenized in 4 volumes of 0.1 M phosphate buffer (pH 7.8); 0.5 ml. of this homogenate were incubated with a solution of TNT (10.5 mg. per 100 cc.) (2.0 ml.) at 37°C. in N₂ for 15 minutes. Initial TNT concentration: 8.4 mg. %

TISSUE	FINAL TNT CONC., mg. %	MG. TNT REMOVED PER GM. DRY TISSUE
Liver. . .	3 8	5.7
Kidney	4.3	5 1
Diaphragm	7 0	1 8
Brain..	7 9	0 6
Spleen	8 25	0 25
Liver previously heated at 80° for 15 minutes	8 4	0
Kidney previously heated at 80° for 15 minutes	8.4	0

anaerobic than under aerobic conditions (table 3). This removal does not require the intact structure of the cell, since TNT disappears also after incubation with homogenized tissues (table 4). The ability of tissues to remove TNT from their environment is lost by heating the tissues at 80° for 15 minutes.

These observations suggest that the removal of TNT by the tissues involves an enzymatic reduction of the compound. Accordingly, an attempt was made to study the metabolism of TNT in the tissues.

Effect of diphosphopyridine nucleotide (DPN) on TNT removal by heart extracts. Since pig heart is a good source of respiratory enzymes and since homogenates of this tissue showed a high rate of TNT removal, an eluate of this organ was chosen for this series of experiments. It was found that this preparation removed

TABLE 5

Effect of DPN on TNT removal and amine formation by heart extracts

Anaerobic incubation of an eluate of pig heart (0.5 ml.) with a solution of TNT (10 mg. per 100 cc.) (1.0 ml.) at 37°C. for 10 minutes (pH 8.4).

SUBSTRATE (FINAL CONC: 0.05 M)	DPN ADDED MG.	MICROGRAMS TNT REMOVED	MICROGRAMS AMINE FORMED
		0	0
	0.2	0	0
Sodium lactate		0	0
Sodium lactate	0.2	87	16
Sodium succinate		0	0
Sodium succinate	0.2	38	12
Sodium malate		0	0
Sodium malate	0.2	81	15

TABLE 6

Removal of TNT by crude succinic dehydrogenase

Anaerobic incubation of the enzyme preparation (0.5 ml.) with a solution of TNT (11.4 mg. per 100 cc.) (1.0 ml.). (37°, 10 min.)

SODIUM SUCCINATE CONCENTRATION	SODIUM LACTATE CONCENTRATION	DPN ADDED MG.	pH	MICROGRAMS TNT REMOVED	MICROGRAMS AMINE FORMED
0.05 mol.			7.4	0	0
0.05 mol.			8.4	2	0.5
	0.05 mol.	0.2	7.4	51	9
	0.05 mol.	0.2	8.4	90	16
0.05 mol.		0.2	7.4	14	4
0.05 mol.		0.2	8.4	26	7

TNT only in the presence of both DPN and a substrate such as lactate, malate or succinate (see table 5; in this and the following tables representative experiments are recorded). The pH optimum for this reaction is 8.4. Neither the addition of the substrates alone nor of DPN alone resulted in the disappearance of TNT. Observations of the same character were made with a crude succinic dehydrogenase preparation (table 6). With both enzyme preparations lactate and malate were more effective substrates than succinate.

In confirmation of the observations of Westfall (6), it was found that TNT removal is associated with the production of a diazotizable substance, indicating the formation of an aromatic amine. However, under these experimental conditions, only one-third to one-fifth of the TNT removed could be accounted for by the formation of an amine. More detailed observations referring to this phenomenon will be described below.

Effect of cysteine on TNT removal by heart extracts. Cysteine was found to increase TNT removal, when small amounts of the enzyme preparation were

TABLE 7

Effect of cysteine on the removal of TNT by an extract of pig heart

Anaerobic incubation of various volumes of heart extract with a solution of TNT (10.0 mg. per 100 ml.) (1.0 ml.) at 37°C. for 10 min. (pH 8.4). Total volume: 2.0 ml. Each mixture contains 0.2 mg. DPN and sodium lactate (0.054 M).

HEART EXTRACT	CYSTEINE CONCENTRATION	MICROGRAMS TNT REMOVED
ml.	M	
0.4		80
0.4	0.01	82
0.2		28
0.2	0.01	47
0.1		6
0.1	0.01	26
0.05		0
0.05	0.05	13

TABLE 8

Effect of cysteine on TNT removal by extract of pig heart after fractionation with ammonium sulfate (15 per cent)

Anaerobic incubation of extract (0.5 ml.) with a solution of TNT (10.0 mg. per 100 ml.) (1.0 ml.) at 37°C. for 10 min. (pH 8.4).

CYSTEINE CONCENTRATION	DPN mg.	SODIUM LACTATE CONCENTRATION	MICROGRAMS TNT REMOVED
0.01 M		0.05 M	0
0.01 M	0.2		0
	0.2	0.05 M	31
0.01 M	0.2	0.05 M	65

used (table 7), or when the eluate was further purified by fractionation with ammonium sulfate (table 8). Cysteine cannot act as a substrate since addition of cysteine and DPN without lactate did not result in a removal of TNT (table 8).

Removal of TNT by Straub's flavoprotein. Use of this system gave results indicating that DPN acts as a hydrogen carrier in the reduction of TNT. Since flavoproteins act as hydrogen acceptors from pyridine nucleotides and as hydrogen donors to higher oxidation-reduction systems, it was tested whether hydrogen is transferred from flavoproteins to TNT. It was found that a system containing

as its sole components reduced DPN and Straub's flavoprotein caused TNT to disappear (table 9). No formation of hydroxylamine or amine took place under these conditions. Reduced DPN in the absence of the flavoprotein was much less effective.

Reduction of TNT to hydroxylamino-dinitrotoluene by xanthine oxidase. Addition of another, though less purified flavoprotein, xanthine oxidase, also led to the disappearance of TNT. Hypoxanthine and xanthine were effective substrates. With this system TNT removal resulted in the formation of a hy-

TABLE 9

Removal of TNT by Straub's flavine enzyme

Anaerobic incubation with a solution of TNT (10.0 mg per 100 ml) (1.0 ml) for 10 min at 37°C Total volume 2 ml (pH 8.0)

REDUCED DPN MG	FLAVINE ENZYME ML	MICROGRAMS TNT REMOVED	
3.5		4	7
	0.1	0	0
3.5	0.1	94	97

TABLE 10

Removal of TNT by xanthine oxidase

Anaerobic incubation of a solution of TNT (10.0 mg per 100 ml) (1.0 ml) with xanthine oxidase (0.3 units) and xanthine (1.0 mg) Total volume 2.0 ml (pH 7.6)

INCUBATION TIME (37°C) MIN	MICROMOLES TNT REMOVED	MICROMOLES OF HYDROXYLAMINE FORMED
2	0.11	0.075
4	0.19	0.14
5	0.24	0.19
6	0.29	0.28
8	0.34	0.34
10	0.37	0.38
20	0.41	0.41
30	0.42	0.41
30	0	0
(No xanthine oxidase)		
30	0	0
(No xanthine)		

droxylamine (table 10). After very short incubation only a part of the TNT removed could be accounted for by the formation of the hydroxylamine, but on further incubation almost all the TNT was converted to hydroxylamine. No amine formation was observed with this enzyme.

Reduction of TNT to amino-dinitrotoluene by liver extracts. While the removal of TNT by flavoproteins did not result in the complete reduction of a nitro to an amino group, this was the case with tissue homogenates, heart extracts (tables 5 and 6) and extracts of acetone-treated pig livers (table 4). Only when

incubation was continued after TNT was no longer being removed could the amount of amine formed be completely accounted for by the removal of TNT (table 11). It is evident, therefore, that the enzymatic reduction of TNT proceeds in at least two steps, the first consisting in the formation of an intermediary compound (or compounds), the second in the reduction of the latter to an amine. The second phase of this reduction proceeds at a slower rate than the first phase, since the amine formation lags behind the removal of TNT and increases only gradually after prolonged incubation.

Isolation of 4-amino 2,6-dinitrotoluene after incubation of TNT with an extract of pig liver. Pig liver extract (600 ml.) was incubated, in an atmosphere of nitrogen at 37°C., with a solution (3000 ml.) containing 12.6 mg. of TNT per 100 ml. When all TNT had disappeared, 96 per cent of it could be accounted for by formation of the amine. The mixture was extracted with ether ($\frac{1}{2}$ volume) after addition of ethyl alcohol ($\frac{1}{10}$ volume) in large separatory funnels. After

TABLE 11

Enzymatic conversion of TNT to amino-dinitrotoluene by acetone extract of liver

Anaerobic incubation of acetone extract of pig liver (0.3 ml.) with a solution of TNT (10.0 mg. per 100 ml.) (1.0 ml.). Total volume: 2.0 ml. (pH: 8.4, 37°C.)

DURATION OF INCUBATION (37°C. MIN.)	LIVER EXTRACT #1		PER CENT OF AMINO-DINITRO- TOLUENE ACCOUNTED FOR BY TNT REMOVAL	LIVER EXTRACT #2		PER CENT AMINO-DINITRO- TOLUENE ACCOUNTED FOR BY TNT REMOVAL
	Micromoles TNT removed	Micromoles amino-dinitro- toluene formed		Micromoles TNT removed	Micromoles amino-dinitro- toluene formed	
10	0.12	0.02	17	0.09	0.03	33
20	0.17	0.04	24	0.16	0.07	44
40	0.22	0.07	32	0.26	0.12	46
60	0.26	0.10	38	0.33	0.17	52
90	0.30	0.14	47	0.36	0.23	64
120	0.32	0.19	60	0.38	0.29	76
180	0.34	0.27	80	0.40	0.38	92
240	0.35	0.34	97	0.41	0.40	97

one extraction only 4 per cent of the amine originally present remained in the aqueous layer. The upper layer was centrifuged. It separated in three phases. The upper phase, a clear, dark-yellow fluid was siphoned off; the intermediary layer, a whitish yellow mass, was filtered off from the lower aqueous phase, extracted with more ether, centrifuged and then combined with the upper phase. The ether extracts were treated with anhydrous sodium sulfate and then evaporated to dryness in an atmosphere of nitrogen under reduced pressure. The residue was dissolved in hot carbon tetrachloride; after addition of charcoal the solution was heated again and filtered. On cooling, small yellow-orange irregular needles crystallized out. After standing overnight in the refrigerator, the crystals were filtered off, dried, then suspended in boiling water and the residue filtered and dried. The material was then recrystallized once from chloroform and three times from benzene. During the last two crystallizations,

regularly shaped needles separated out. The compound isolated in this manner melted at 170–171°C. This melting point did not change on further recrystallization. When mixed with synthetic 4-amino 2,6-dinitrotoluene (Mp. 170–171°C.), an identical melting point was observed. Titration with titanous chloride revealed that the compound had two nitro groups (assuming the molecular weight of amino-dinitrotoluene). Elementary analysis: C: 42.75%, H: 3.57%, N: 21.25% (calculated for $C_7H_7N_3O_4$: C: 42.7%, H: 3.55%, N: 21.3%). Accordingly, the compound isolated as a result of the enzymatic reduction of TNT by liver extracts has been identified as 4-amino-2,6-dinitrotoluene.

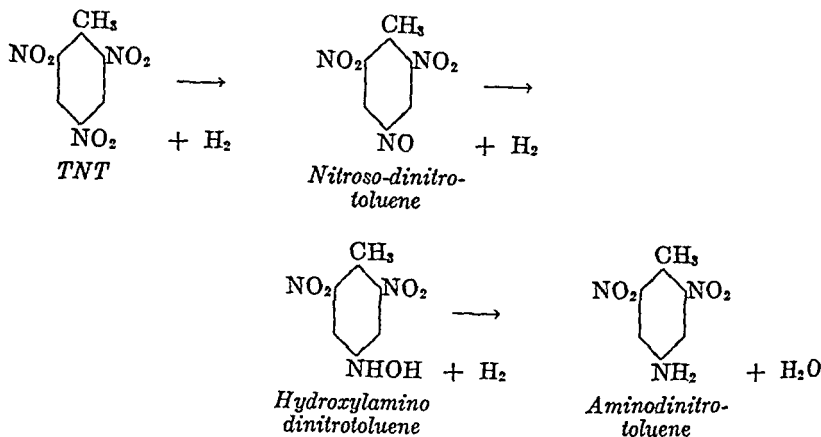
Isolation of 2,2'-6,6'-Tetranitro-4-azoxytoluene after incubation of xanthine oxidase with TNT. A solution of TNT (14.3 mg. per 100 ml.; 2000 ml.) was incubated with xanthine oxidase (80 units) and xanthine (750 mg.) at 37° in an atmosphere of nitrogen until all TNT had been removed and could be accounted for by hydroxylamine formation. No azoxy compound could be detected in the mixture. The mixture was extracted with ether and evaporated to dryness in a similar manner as in the isolation of the amine. The dry residue was dissolved in acetone. On adding alcohol a whitish yellow amorphous precipitate separated. It was filtered and crystallized from an alcohol-acetone mixture. After two more recrystallizations, very pale, yellowish needles were obtained which melted at 212–213°C. This melting point did not change on further recrystallization. When mixed with synthetic 2,2'-6,6'-4-azoxytoluene (Mp. 212–213°C.) the melting point did not change. Elementary analysis: C: 41.4%, H: 2.58%, N: 20.85% (calculated for $C_{14}H_{10}O_8N_6$: C: 41.4%, H: 2.45%, N: 20.7%). The compound isolated is therefore, 2,2'-6,6'-tetranitro-4-azoxytoluene.

DISCUSSION. The isolation of 2,2'-6,6'-tetranitro-4-azoxytoluene from a solution in which hydroxylamine, but no azoxy-compound, could be detected requires some comment. Aromatic hydroxylamines are extremely labile and are very readily converted into azoxy-compounds. Although no drastic procedure during the isolation was used and particular care was taken to avoid oxidation by using peroxide-free ether and by evaporating the ether extract under an atmosphere of nitrogen, it is most probable that the azoxy-compound was formed during the isolation procedure and was not originally formed by the action of xanthine oxidase on TNT.

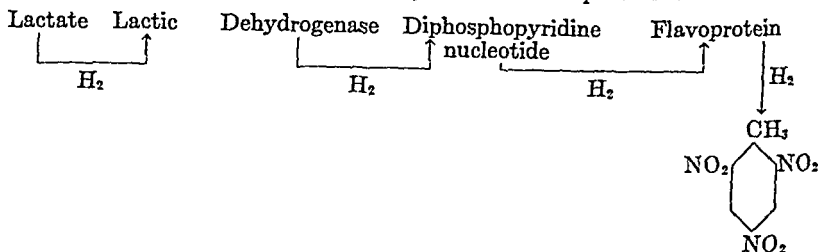
The isolation of 4-amino-2,6 dinitrotoluene as a result of the incubation of TNT by liver extracts demonstrates that the metabolism of TNT *in vitro* consists in the reduction of one nitro group to an amino group. Reduction to the amine does not proceed in one but in several steps, since TNT is removed at a much faster rate than the amine is formed. Therefore, the hydroxylamino-compound accumulates during the first phase of the reduction of TNT. Each of these steps in the reduction of TNT is catalyzed by a different hydrogen-transferring system. The reduction of TNT is initiated by flavoproteins, but these enzymes do not complete the reduction to the amine. Straub's flavoprotein can apparently reduce TNT only to the nitroso stage. This is analogous to an enzyme system activated by di- and triphosphopyridine nucleotides, described by

Hoagland and Ward (17) in *Hemophilus influenzae*, which reduces inorganic nitrate to nitrite. Xanthine oxidase, another flavoprotein, catalyzes the reduction of TNT to the hydroxylamine stage.

The stepwise reduction of TNT by animal tissues can be pictured in the following manner:



The fact that eluates of pig heart reduce TNT only in the presence of DPN and a suitable substrate (lactate, malate) shows that the first step in the reduction of TNT in the tissues consists in the transfer of hydrogen from the substrate through a specific dehydrogenase, DPN and flavoprotein to TNT:



Our observations that succinic dehydrogenase cannot catalyze this reduction are in apparent disagreement with the findings of Westfall (15), who believed that TNT is reduced to amino-dinitro-toluene by succinic dehydrogenase. This apparent discrepancy could be explained by the fact that DPN may not have been completely removed from Westfall's succinic dehydrogenase preparation. In this case added succinate would be oxidized to fumarate which in turn is converted to malate by the action of fumarase. Malate in presence of DPN would then function as a hydrogen donor for TNT. Such a series of reactions was observed (tables 5 and 6). The stepwise reduction of TNT might explain

the effect of this compound on tissue respiration. The decrease in oxygen uptake occurs only after a large amount of TNT originally present in the medium has already been removed (tables 1 and 2). Thus, either TNT diffuses only at a very slow rate into the cells or it is converted into a compound inhibiting tissue respiration. In this case the metabolism of TNT *in vitro* would not represent a "detoxication," since it at first gives rise to a compound more toxic than TNT itself.

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SUMMARY

1. TNT is converted by slices of muscle and of liver into a compound which inhibits tissue respiration; this compound is then further metabolized with the resultant formation of a product having no inhibitory effect on the oxygen uptake of tissues.

2. TNT removal by tissue slices and homogenates is more rapid under anaerobic than under aerobic conditions.

3. The presence of DPN is essential for the removal of TNT by heart extracts. Cysteine increases the rate of the removal of TNT.

4. TNT is removed by a system containing reduced DPN and a highly purified flavoprotein (Straub). Nitroso-dinitrotoluene is the probable product of this reduction.

5. TNT is reduced by partially purified xanthine oxidase to hydroxylamino-dinitrotoluene.

6. 4-amino-2,6-dinitrotoluene was isolated as the end product of the metabolism of TNT by liver extracts.

7. The metabolism of TNT in animal tissues consists in a stepwise reduction of one nitro group. The first step is brought about by a transfer of hydrogen from flavoproteins to the nitro group. Hydroxylamino-dinitrotoluene accumulates because the last step, reduction to the amine, proceeds at a slower rate than the initial phase of the metabolism of TNT.

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THE SYNERGISM BETWEEN THE BARBITURATES AND ETHYL ALCOHOL

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In 1934 Carriere, Huriez and Willoquet (1) conducted a study of the combined administration of alcohol and Gardenal (phenobarbital) to rabbits and reported an antagonism to exist between these two substances; they found that alcohol delayed the onset of the barbiturate anesthesia and reduced its toxicity. Allegrì (2), using rabbits, dogs and guinea pigs, partially confirmed these results; in many of his experiments, however, he found alcohol to have no analeptic effect upon animals treated with Gardenal or with Veronal (barbital).

In direct contrast to the conclusions of Carriere and his associates, several authors report a synergism to exist in the action of alcohol and the barbiturates. Olszycka (3, 4) demonstrated in the mouse and the rat a potentiation of the action of ethyl-butyl barbituric acid by the presence of alcohol as measured in terms of duration of sleep. Dille, Linegar and Koppányi (5) in connection with experiments in which alcoholic solutions of barbital were injected into the carotid artery of the dog, made the statement that methyl alcohol and barbital were two-fold synergists, "methyl alcohol possessing not only a central depressant action, in addition to that of barbital, but also enabling the brain to bind larger amounts of di-ethyl barbituric acid." In a study of the synergism of ethyl alcohol and sodium pentobarbital, Dille and Ahlquist (6) found the average potentiation of alcohol depression by sodium pentobarbital to be greater with small doses than with large; they found the rate of elimination of ethyl alcohol to be unaffected by the presence of pentobarbital and conversely the rate of elimination of pentobarbital to be unaffected by alcohol. Jetter and McLean (7) reported that in rats receiving alcohol intraperitoneally and phenobarbital subcutaneously, a maximum sub-lethal dose of each drug produced death regularly when given in combination. Seeberg and Dille (8) showed that barbital administered as an elixir, because of its alcohol content, produced more profound depression than that warranted by the blood barbiturate level.

The widespread use of both alcohol and the barbiturates would appear to make important a more thorough knowledge of the effects that may ensue when they are administered jointly. That this combination can be of clinical significance has been stressed by Jetter and McLean (7). Thus they found the cause of death in three persons to be the result of the ingestion of a barbiturate plus alcohol, and not of either substance alone. In this connection there naturally arises the query as to the possibility of special problems being encountered in the use of barbiturate anesthesia in patients under the influence of alcohol. Because of the apparent practical importance of the matter as well as for purely scientific

reasons, the present laboratory experiments were undertaken with the hope of adding further to the information on alcohol-barbiturate synergism.

EXPERIMENTAL. In the present study the problem of alcohol-barbiturate synergism has been investigated from several aspects: A. the toxicities for mice of three different barbiturates in aqueous and alcoholic solutions; B. the synergism in dogs of alcohol and pentothal as measured by duration of pentothal effect and the change in its anesthetic and lethal doses; and also the difference in the anesthetic effects when the alcohol and pentothal were given in different time relationships; C. the blood levels of alcohol and barbiturate in dogs when ethyl alcohol and sodium barbital were given separately and in combination; and finally, D. the effectiveness of picrotoxin as an antidote to the combined effects of alcohol and barbiturate depression in rabbits.

For the determination of comparative blood barbiturate levels following administration of barbital, the isopropylamine method described by Koppanyi, Dille, Murphy and Krop (9) seemed suitable and was employed after adaptation for use with small amounts (2 cc.) of blood.

Blood alcohol values were established by oxidation with a standard silver chromate-potassium dichromate reagent, the excess oxidizing reagent being determined with standard sodium thiosulfate after addition of potassium iodide (10).

A. Toxicity of seconal, pentobarbital and barbital in aqueous and alcoholic solution: These experiments, designed to compare the toxicities of barbiturates when given in aqueous and alcoholic solutions, involved the oral administration of the drugs to mice. Sodium seconal, sodium pentobarbital and sodium barbital were selected because of the range in duration of action among them. Solution concentrations were adjusted so that a uniform total volume of 30 cc./Kg. was maintained throughout.

The LD50 of sodium seconal was established as 140 mgm./Kg.; the LD50 of sodium pentobarbital was found to be 180 mgm./Kg.; that for sodium barbital was 900 mgm./Kg. The oral LD50 for 95 per cent alcohol was determined as 11.1 cc./Kg.

A single experiment was carried out in which the LD50 for sodium seconal as given in alcoholic solution was determined. When sodium seconal was mixed with alcohol in a dilution such that the mice received 4.2 cc. 95 per cent alcohol/Kg., the LD50 for sodium seconal became 105 mgm./Kg., as compared with 140 mgm./Kg. in the absence of alcohol.

For the major portion of the series modification of the mortality rate for a given dose of the barbiturate was studied. In doing this a group of mice was divided into six lots of 50 mice each. Three lots received the barbiturates in aqueous solution, and three received them in alcoholic solution. The dose of each barbiturate was approximately 70 per cent of its LD50, namely (a) sodium seconal 100 mgm./Kg., (b) sodium pentobarbital 130 mgm./Kg. and (c) sodium barbital 650 mgm./Kg. The amount of alcohol chosen was 4.2 cc. of 95 per cent alcohol per Kg., a definitely sublethal dose (20 mice were given this amount of alcohol alone and were neither killed nor anesthetized by it).

The results of the experiments are shown in table 1. In each case the addition of alcohol increased the toxicity of the barbiturate as measured by the per cent mortality. In the case of sodium seconal this increase was from 18 per cent

mortality among animals receiving sodium seconal in water to 90 per cent among those receiving it in alcoholic solution. In the case of sodium pentobarbital the mortality rose from 16 to 80 per cent, and in the case of sodium barbital it increased from 30 per cent to 66 per cent. Apparently the toxicity of the shorter acting hypnotics was influenced more intensely than that of the longer acting one used.

B. *Pentothal given intravenously in the presence of alcohol:* A series of studies was made on dogs concerning the synergistic action of alcohol with the short-acting barbiturate, sodium pentothal. The anesthetic was injected intravenously,

TABLE 1

Effect of alcohol on barbiturate toxicity and duration of anesthesia in mice

DRUG	DOSE	NUMBER OF ANIMALS	NUMBER DEAD	PER CENT MORTALITY	TIME OF DEATH*	TIME OF RECOVERY
					minutes	minutes
Sodium seconal (aqueous soln)	100 mgm /Kg	24 26	9 0	37.5 0	28.8	234.4
Sodium seconal (alcoholic soln)	100 mgm /Kg + 4.2 cc 95% alc /Kg	24 26	22 23	91.3 88.5	22.1 43.7	911.3
Sodium pentobarbital (aqueous soln)	130 mgm /Kg	50	8	16	51.3	289.6
Sodium pentobarbital (alcoholic soln)	130 mgm /Kg + 4.2 cc 95% alc /Kg	50	40	80	44.9	470.5
Sodium barbital (aqueous soln)	650 mgm /Kg	50	15	30	145.4	1837
Sodium barbital (alcoholic soln)	650 mgm /Kg + 4.2 cc 95% alc /Kg	50	33	66	141.4	1331
Alcohol	4.2 cc 95% alc /Kg	20	0	0		

* Time of death of animals dying in 48 hours or less

and the alcohol was given orally thus simulating conditions which might be encountered clinically. Two dose levels of alcohol were used, 1.5 cc. and 3 cc. of 95 per cent alcohol per Kg. body weight. The higher of these two doses, when given alone, produced visible signs of intoxication, such as staggering gait, but was not sufficient to produce loss of consciousness. Effects from the smaller dose of alcohol were proportionally less. Observations were made on the anesthetic and lethal doses of sodium pentothal when given alone and when administered in the presence of alcohol.

The procedure was as follows. The normal minimal anesthetic dose of sodium pentothal was determined for each of 10 dogs of about equal weight; this was done by injecting a 1 per cent solution of the drug intravenously at the rate of 1 cc. per 30 seconds until disappearance of the corneal reflex. The rate of injection was carefully controlled since variation in this respect may influence the results (11). One week or more later five of the dogs were injected again, this time two hours after the oral administration of 1.5 cc. of 95 per cent alcohol per Kg., diluted 1:10 with water. After the amount of sodium pentothal necessary to abolish the corneal reflex was noted the injection was continued until the total reached the amount that had been necessary to produce anesthesia in that animal under normal circumstances. Thus the duration of anesthesia following a given dose of sodium pentothal when administered after alcohol could be compared to the duration of anesthesia following the same dose of sodium pentothal in the absence

TABLE 2

A comparison of the effect of sodium pentothal administered in the absence of alcohol and 2 hours after administration of alcohol

(Each figure is the average for five dogs)

PRELIMINARY TREATMENT	ANESTHETIC DOSE OF SODIUM PENTOTHAL mgm /Kg	EFFECT OF NORMAL DOSE OF PENTOTHAL	
		Loss of corneal reflex minutes	Loss of movement minutes
None	10.9	1.4	11.4
95% Alcohol 1.5 cc./Kg	6.9	13.2	22.0
None	10.2	1.8	10.8
95% Alcohol 3.0 cc./Kg.	5.3	27.2	51.4

of alcohol. The five remaining dogs were treated similarly except that the dose of alcohol given them was higher—3 cc. of 95 per cent alcohol per Kg. The results of this study are shown in table 2.

The administration of 1.5 cc. per Kg. of alcohol before injection of the sodium pentothal apparently influenced the potency of the barbiturate; the anesthetic dose was reduced from 10.9 mgm./Kg. to 6.9 mgm./Kg., a decrease of 36.2 per cent. The previous administration of 3 cc. of alcohol per Kg. produced even more striking results, the anesthetic dose being reduced from 10.2 mgm./Kg. to 5.3 mgm./Kg., a decrease of 48.2 per cent.

The duration of anesthesia resulting from a fixed dose of the drug was prolonged by the presence of alcohol. The corneal reflex which returned normally in 1.4 minutes in the first group of dogs was delayed until 13.2 minutes after onset of anesthesia when the dose of alcohol was 1.5 cc./Kg., and in the second group of dogs, given 3 cc./Kg. of alcohol, its return was delayed from 1.8 minutes until

27.2 minutes after the end of injection. Duration of anesthesia was also measured in terms of the lapse of time between injection of the drug and the recovery of voluntary movement. In the case of the dogs given the smaller dose of alcohol this recovery required 11.4 minutes normally and 22.0 minutes when alcohol was present; the dogs used with the larger dose of alcohol recovered movement after 10.8 minutes normally and after 51.4 minutes when alcohol was present.

The effect of alcohol upon the lethal dose of sodium pentothal was also investigated. Two groups of five dogs each were used, the average anesthetic dose of sodium pentothal of each group having been determined as 10.6 mgm./Kg. One group was given alcohol orally (3 cc. 95 per cent alcohol/Kg., diluted 1:10). All dogs were injected with 1 per cent sodium pentothal until cessation of respiration; this was done by injecting an anesthetic dose rapidly and the remainder of the drug at the rate of 1 cc. per 30 seconds. The dogs given alcohol were injected after 2 hours when the average blood level of alcohol was 152.7 mgm./100 cc.

The lethal dose of sodium pentothal for the control group was found to be 68.7 mgm./Kg. That for the group under the influence of alcohol was 40.6 mgm./Kg., representing a decrease in lethal dose of 40.9 per cent.

An attempt was made to demonstrate the variation in activity of sodium pentothal, as influenced by alcohol, according to the length of time that had elapsed since ingestion of the alcohol, and therefore according to the blood level of alcohol at the time of the injection of the anesthetic.

Three groups of six dogs were used, and the normal values for the anesthetic dose of pentothal and the duration of its effect determined for each dog under normal circumstances. The dogs of the first group were given 3 cc. of 95 per cent alcohol per Kg. orally, and one hour thereafter were injected intravenously with the 1 per cent solution of sodium pentothal; the anesthetic dose was noted and the injection continued until the originally determined anesthetic dose of pentothal had been given. The blood alcohol level was determined at the time of the injection. Dogs in the second group were injected with sodium pentothal two hours after alcohol had been given; those of the third group were injected three hours after alcohol.

A comparison of the pentothal reaction at these three time intervals is made in table 3. The anesthetic dose of pentothal one hour after alcohol was 58.8 per cent of the normal dose; two hours after alcohol it was 53.0 per cent of the normal dose; three hours after administration of alcohol the anesthetic dose was 53.3 per cent of the normal dose. The duration of absence of the corneal reflex after a fixed dose of sodium pentothal varied inversely with the length of time after administration of alcohol. This reflex was absent 33.5 times as long when sodium pentothal was given one hour after alcohol as it was after sodium pentothal alone; the corneal reflex was absent for 14.0 times as long when sodium pentothal was given two hours after alcohol as it was in the absence of alcohol; when sodium pentothal was given three hours after alcohol the reflex was absent for a period 11.3 times as long as when pentothal alone was given. Duration of anesthesia measured in terms of loss of voluntary movement varied in the same direction but

was less strikingly different from the normal figures; the ratio of this duration to its corresponding normal value was 6.5 times, 5.1 times and 4.2 times at one, two and three hours respectively after the administration of alcohol.

Thus while the minimal anesthetic dose of pentothal was markedly diminished by the presence of alcohol, the diminution in necessary dosage remained relatively constant at the various intervals studied following alcohol administration. With respect to duration of anesthesia, however, this was definitely more prolonged when the interval between alcohol administration and pentothal injection was one

TABLE 3

Effect of sodium pentothal on dogs at 1, 2 and 3 hours after administration of alcohol (3 cc 95% alcohol/Kg)

	GROUP A (6 DOGS)		GROUP B (6 DOGS)		GROUP C (6 DOGS)	
	Normal	1 hr after alcohol	Normal	2 hrs. after alcohol	Normal	3 hrs after alcohol
Blood level of alcohol (mgm./100 cc)		187 5		193 2		171.4
Anesthetic dose of sodium pentothal mgm /Kg	13 6	8 0	10 6	5 6	12.7	6 8
Duration of loss of corneal reflex (min.) after normal anesthetic dose of sodium pentothal	1 3	43 5	1.9	26 5	2	22 5
Duration of loss of movement (min) after normal anesthetic dose of sodium pentothal	11 0	72 0	10.7	59 0	8 1	33.7

TABLE 4

The effect of alcohol upon depression produced in dogs by sodium barbital
(Figures are averages of 6 experiments)

	ONSET OF ANESTHESIA	DURATION OF LOSS OF CORNEAL REFLEX
	minutes	minutes
Sodium barbital 180 mgm /Kg intra-venously	35 3	104 5
Sodium barbital 180 mgm /Kg intra-venously plus 95% alcohol 3 cc /Kg orally	83 8	325.3

hour as compared to intervals of two or three hours, this despite approximate equal blood alcohol levels.

C. Disposition of alcohol and barbiturates in the body: Six dogs were used in a study of barbiturate depression designed to investigate the influence, if any, of the presence of sodium barbital on the absorption and elimination of alcohol, and conversely the influence of alcohol upon the disposition of sodium barbital. On separate occasions these dogs were given (a) alcohol by mouth, (b) sodium barbital by vein, and (c) sodium barbital injected one hour after oral administration

of alcohol. The amount of alcohol used was 3 cc./Kg.; the dose of sodium barbital was 180 mgm./Kg., a dose which produced light anesthesia.

Table 4 shows the effect of alcohol upon the extent of depression in these dogs. Considerable variation in susceptibility of individual animals was noted.

In all instances the onset of anesthesia in these animals was delayed by the presence of alcohol although the duration of anesthesia was materially increased. This observation is in keeping with the statement of Carriere, Huriez and Willoquet (1) that the onset of phenobarbital anesthesia was delayed by alcohol, but disagrees with their findings in regard to toxicity.

The blood level of alcohol was followed for about seven hours after its administration. The level of blood barbiturate was determined at intervals for four to five hours after injection of sodium barbital. The composite records of these

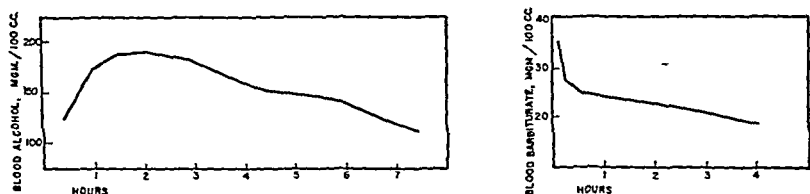


FIG. 1. BLOOD ALCOHOL AND BARBITURATE LEVELS (AVERAGE FOR 6 DOGS) WHEN EACH DRUG WAS GIVEN SEPARATELY

Alcohol 3 cc. 95%/Kg.; sodium barbital 180 mgm./Kg.

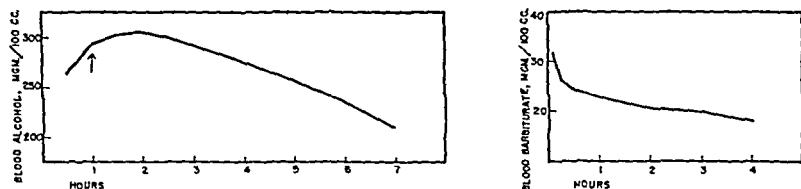


FIG. 2. BLOOD ALCOHOL AND BARBITURATE LEVELS (AVERAGE FOR 6 DOGS) WHEN DRUGS WERE GIVEN SIMULTANEOUSLY; ARROW INDICATES INJECTION OF SODIUM BARBITAL
Doses as for fig. 1.

determinations are shown in figs. 1 and 2. The highest blood alcohol level was reached at about 2 hours; this agrees with the data published by Newman (12). It is obvious that the alcohol curve produced in the presence of barbital does not differ in form from the curve made when alcohol alone was given, although, presumably because of animal variation, the actual values do. The curves representing the amount of barbital present in the blood were also practically identical. The results confirm the work of Dille and Ahlquist (6).

D. *Picrotoxin as an antidote to the alcohol barbiturate combination*: Since picrotoxin has been found to be a relatively efficient antidote in barbiturate poisoning (13), but ineffective in alcohol depression (14), an investigation of its effectiveness as an analeptic to the combination of a barbiturate and alcohol was conducted. Rabbits were used as experimental animals. The barbiturate chosen was sodium

pentobarbital which was given intravenously in a dose of 30 mgm./Kg. The dosage of alcohol selected was 1.5 cc. of 95 per cent, appropriately diluted for intravenous administration; this amount given alone caused no anesthesia but produced symptoms of intoxication such as incoordination of movement. Picrotoxin was used in 2 mgm./Kg. doses.

Five groups of 10 rabbits each were used. These were injected with the following: (a) alcohol, (b) sodium pentobarbital, (c) alcohol plus sodium pentobarbital, (d) sodium pentobarbital plus picrotoxin and (e) alcohol plus sodium pentobarbital plus picrotoxin. In all cases three injections were made, spaced at 10 minute intervals; a suitable amount of Ringer's solution was injected when

TABLE 5

Picrotoxin as an antidote to alcohol sodium pentobarbital combination in the rabbit All drugs administered intravenously
(Figures are averages of 10 animals)

	DURATION OF ANESTHESIA
	minutes
Ringer's Solution Sodium pentobarbital 30 mgm /Kg Ringer's Solution	100 2
Alcohol 1 5 cc /Kg Ringer's Solution Ringer's Solution	0
Alcohol 1 5 cc /Kg Sodium pentobarbital 30 mgm /Kg Ringer's Solution	183 8
Ringer's Solution Sodium pentobarbital 30 mgm /Kg Picrotoxin 2 mgm /Kg	37 1
Alcohol 1 5 cc /Kg Sodium pentobarbital 30 mgm /Kg Picrotoxin 2 mgm /Kg	135 8

no drug was to be used. The duration of anesthesia was measured in each of the animals by noting the time from the injection of sodium pentobarbital until recovery of the righting reflex.

The duration of anesthesia in each of the the groups is tabulated in table 5. Sodium pentobarbital alone produced loss of the righting reflex for 100.2 minutes; previous administration of alcohol increased the duration of anesthesia by 83.6 minutes or 83.4 per cent. The normal duration of anesthesia upon injection of sodium pentobarbital was reduced by the administration of picrotoxin to 37.1 minutes, a decrease of 63.1 minutes or 63.0 per cent. The injection of both alcohol and sodium pentobarbital produced anesthesia of 183.8 minutes' duration;

this time was reduced by administration of picrotoxin to 135.8 minutes, a decrease of 48.0 minutes or 26.1 per cent.

Thus it appears that picrotoxin is less efficient as an antidote to the depression produced by the combination of sodium pentobarbital and alcohol than it is against the action of the barbiturate alone.

Insofar as the duration of anesthesia may be considered an index to depression, the results of this experiment on the relative analeptic activity of picrotoxin indicate that possibly a potentiative, rather than a simple additive, type of synergism exists between the barbiturates and alcohol.

SUMMARY

1. The LD50 of sodium seconal given orally to mice was found to be 140 mgm./Kg.; when administered in combination with alcohol (4.2 cc. 95 per cent per Kg.) the LD50 was lowered to 105 mgm./Kg.

2. The presence of 4.2 cc. of 95 per cent alcohol/Kg. materially increased the per cent mortality resulting from standard oral doses of sodium seconal, sodium pentobarbital, and sodium barbital in mice.

3. The anesthetic dose of sodium pentothal for dogs was reduced from 10.9 to 6.9 mgm./Kg. by previous oral administration of 1.5 cc. 95 per cent alcohol/Kg. and from 10.2 to 5.3 mgm./Kg. by 3 cc. 95 per cent alcohol/Kg. Duration of anesthesia resulting from sodium pentothal injection was greatly increased in the presence of alcohol.

4. The minimal anesthetic dose of sodium pentothal when given one, two and three hours after administration of alcohol were not strikingly different. The duration of anesthesia after a fixed dose of sodium pentothal in the presence of alcohol was greatest when given one hour after alcohol and least when given three hours after administration of alcohol.

5. The onset of anesthesia from injection of sodium barbital appeared to be delayed by the presence of alcohol. The duration of anesthesia resulting from the injection of sodium barbital was materially increased by the presence of alcohol.

6. The disposition in the body of alcohol appeared not to be influenced by the presence of sodium barbital. Conversely, the blood level of sodium barbital was not altered by the presence of alcohol.

7. Picrotoxin was found less efficient as an analeptic in rabbits given sodium pentobarbital plus alcohol than in those given sodium pentobarbital alone.

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THE INABILITY OF VITAMIN B COMPLEX AS LIVER EXTRACT TO PROTECT THE KIDNEY AGAINST THE TOXIC ACTION OF URANIUM NITRATE¹

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Since the demonstration by Leconte (1) in 1854 that the salts of uranium possessed a nephrotoxic action, this substance has been used by numerous investigators to induce a renal injury, to study the sequence in the development of such morphological changes and to attempt a correlation between such physical modifications of structure with certain functional expressions of altered renal effectiveness as shown by changes in both the blood and urine. As such studies have progressed attempts have been made to protect the kidney against the toxic action of certain of the salts of uranium, especially the nitrate and to explain the mechanism by which such a degree of protection could be induced. Finally, with the observation (2) that an acquired resistance of certain segments of the renal nephron to uranium nitrate could be made to develop by repeating such injections, has given emphasis to the influence of the resistance of fixed tissue cells to injury by a change in their chemical constitution with or without a change in their morphology (3, 4).

In 1916 the observation was made in this laboratory (5) that the intravenous use of sodium carbonate would not only protect the kidney of the dog against the acute injury from uranium nitrate but that it would also protect the kidney of such an animal against the toxic action of a general anesthetic body. The first part of this observation, the protection of the kidney against uranium nitrate, was confirmed by Goto (6) in 1917 by his use of sodium bicarbonate in place of the carbonate. Very recently Donnelly and Holman (7) have demonstrated the same order of protection with an apparent furtherance of the process of epithelial repair in the kidney by employing a solution of sodium citrate. In the initial experiments that have been referred to above (2) the observation was made that uranium nitrate in a constant quantity per kilogram was definitely more toxic for the kidney of old animals as contrasted with its toxic expression in young animals and puppies. Not only was the renal injury of a severer order but as these and later experiments demonstrated there was a more marked general disturbance in the older animals than in the younger members of the series in that there was an earlier and more significant disturbance in the ability of such animals to maintain a normal acid-base equilibrium of the blood, a greater degree of glycosuria and a more rapid appearance and greater output of diacetic acid in the urine. In an attempt to explain these observations the hypothesis (5)

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I am indebted to the Lederle Laboratories, Inc., for their courtesy in furnishing the "Vitamin B Complex".

was indulged in that "it is possible that uranium nitrate, as used in the experiments as recorded in this paper, may exert a similar inhibitory effect on the oxidative enzymes of a variety of cells, and that through this action uranium induces histological changes which are common to different cells, such as acute swelling, and causes the appearance in the urine of various abnormal bodies. It would then follow from the facts observed in the experiments that the oxidative capacity of the younger animals is greater than the oxidative capacity of the older animals; for when both types of animals have been given the same amount of uranium per kilogram there is less evidence of its inhibitory effect on processes of oxidation in the young animals than in the old animals. The younger animals show a lower percentage of glucose in the urine, a later appearance of acetone bodies in the urine and as a result of the milder grade of nephritis, there is a reduced output of albumin." As a result of this order of reasoning the possibility presented itself that the use of a series of bodies such as those found in the vitamin B complex might so accelerate and bring to a state of completion intracellular processes of oxidation placed in a state of partial inhibition from the uranium intoxication as to protect in some measure such cells against this order of injury.

TECHNIQUE OF EXPERIMENTS. Twelve female dogs varying in age from 2 to 6 years have been used for experimental purposes. The animals were kept in metabolism cages, fed on Purina Dog Chow and allowed an unrestricted amount of water. Prior to the experimental interference, in order to determine that the animals were normal and later during their course catheterized specimens of urine were examined qualitatively for albumin, glucose, acetone and diacetic acid and in addition studied microscopically. The acid-base equilibrium of the blood was ascertained by determining the carbon dioxide combining power of the plasma and the immediate state of renal function was ascertained by the use of the phenolsulphonaphthalein test. In addition urea nitrogen, non-protein nitrogen and creatinine determinations were made to indicate the degree of sustained renal dysfunction. Six of the 12 animals were used as controls and were given subcutaneously one injection of 3 mgm. of uranium nitrate per kilogram without having received prior to its use or later during the experiment injections of vitamin B complex. The remaining 6 dogs were given intramuscularly 0.25 cc. of the vitamin B complex every other day for 12 injections before the use of uranium nitrate and continued at such intervals during the course of the experiments. Two control animals and 2 animals in which an attempt had been made to induce a protection were sacrificed on the sixth day of the experimental period in order to ascertain by histological studies the degree of renal injury or protection. The following protocols of 2 of these animals, 1 from each group, are representative of the respective groups.

CONTROL ANIMAL. Series K. #4. Weight 12.8 kilograms. The preliminary studies showed that the urine was free from albumin, glucose and diacetic acid. Casts were not present. There was an elimination of 60 per cent of phenolsulphonaphthalein during the first hour. The whole blood gave the following values in mgm. per 100 cc.: urea nitrogen 7.4, non-protein nitrogen 25.4 and creatinine 1.51. The carbon dioxide plasma tension was 48.7. On the second day following the subcutaneous injection of 3 mgm. of uranium nitrate per kilogram the urine contained albumin and glucose but no diacetic acid. The elimination of phenolsulphonaphthalein in an hour period was reduced to 5 per cent. There

as a retention of urea nitrogen to 16.4 mgm., non-protein nitrogen to 52.7 mgm. and no retention of creatinine. The carbon dioxide tension was reduced from 38.7 to 36.9. On the sixth day of the intoxication, at which time the renal injury has usually reached its height, both the amount of albumin and glucose had increased in the urine and at this time diacetic acid was also present. The urine contained numerous granular casts and both red and white blood cells. The first hour output of phenolsulphonephthalein was reduced to a trace. At this period urea nitrogen had increased to 26.9 mgm., non-protein nitrogen to 80.5 mgm. and creatinine to 3.12 mgm. per 100 cc. of blood. The plasma tension of carbon dioxide was reduced to 28.7.

PROTECTED ANIMAL. Series K. #8. Weight 15.7 kilograms. The animal received 6 intramuscular injections of 0.25 cc. per kilogram of vitamin B complex prior to the subcutaneous administration of 3 mgm. of uranium nitrate per kilogram. The vitamin injections were continued on alternate days during the course of the experiment. The normal observations showed the urine to be free from albumin, glucose and diacetic acid. There was a first hour elimination of 64 per cent of phenolsulphonephthalein. The whole blood gave the following values in mgm. per 100 cc.: urea nitrogen 8.7, non-protein nitrogen 27.3 and creatinine 1.64. The carbon dioxide plasma tension was 50.1. On the second day of the uranium intoxication the urine contained both albumin and glucose but was free from ketone bodies. The first hour output of phenolsulphonephthalein was 8 per cent. There was a retention of urea nitrogen to 14.8 mgm., non-protein nitrogen to 56.8 mgm. with no retention of creatinine. The plasma carbon dioxide tension was reduced to 32.9 from a normal of 50.1. On the sixth day of the intoxication the urine showed a marked increase in albumin and glucose and contained diacetic acid. Finely and coarsely granular casts with occasionally a fatty cast were present. The elimination of phenolsulphonephthalein was in the amount of a trace. At this stage of the intoxication urea nitrogen had increased to 30.2 mgm., non-protein nitrogen to 88.7 mgm. and creatinine to 4.7 mgm. per 100 cc. of blood. The plasma carbon dioxide was 24.9.

The gross and histological studies of the kidneys which were obtained on the sixth day of the intoxication from these two groups of animals show no difference in their degree of injurious response to uranium nitrate. The gross appearance is one of a swollen, congested kidney from which the fibrous capsule stripped with ease. Histologically the glomerular capillaries were usually distended with blood without the development of an exudate or an actual hemorrhage into the subcapsular space. The characteristic injury is as usual of an epithelial order and is most marked in the proximal segment of the renal nephron. This injury consists in edema, vacuolar and fatty degenerations and in areas well advanced cellular necrosis. The amount of stainable lipid material in both the descending and ascending limbs of Henle's loops is greatly increased.

CONCLUSIONS

A comparative study of protocols indicate no appreciable quantitative difference in the degree of reaction of the two groups of animals to an intoxication by

uranium nitrate either when this substance is given alone or when it is preceded by the use of vitamin B complex and continued during the course of the experimental periods. There is no evidence that the use of the substance facilitates processes of tissue oxidation which might be indicated by a modification in the glucose and diacetic values in the urine, the tension of plasma carbon dioxide and the degree of edema and the intensity of the lipoid changes in renal epithelium.

Experiments are now in progress in which various factors in the vitamin B complex are being used in sufficient concentration to demonstrate any specific influence which they may possess in influencing the nephrotoxic action of uranium nitrate.

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STUDIES ON DDT (2,2 BIS-PARACHLORO PHENYL-1,1,1, TRICHLOROETHANE): EFFECTS ON OXIDATIVE METABOLISM¹

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In a preliminary study of the effects of 2,2 bis-parachloro phenyl-1,1,1, trichloroethane (DDT) on tissue respiration, it was found that the oxygen consumption of rat liver slices was elevated above that of unpoisoned controls. In view of this, experiments were designed to investigate the effects of DDT on the overall metabolism of the rat and on the *in vitro* metabolism of certain tissues.

The effect of DDT on the basal metabolic rate of the albino rat was studied on a group of 10 animals. They were fed 0.1 per cent DDT in the diet for a period ranging from 4 to 12 days (average of 8 days). The average amount of DDT consumed was 48.4 mg./kg. per day. A daily record of the body weight, food intake and water consumption was kept. The basal metabolic rate was determined at frequent intervals after the method of Davis and Van Dyke. (1) The animals were anesthetized with sufficient pentobarbital to abolish tremors during the determination. This usually required a dose of 30 to 50 mg. per kg. according to the severity of the symptoms.

The average basal metabolic rate of these animals rose sharply reaching a peak 23 per cent above the control on the third day. The average food and water intake was found to increase in the same fashion, roughly approximating the increase in the basal metabolic rate. The body weight declined slightly over the course of the experiment. The results are shown in figure 1.

At a time when the basal metabolic rate was elevated, and signs of poisoning pronounced, the animals were sacrificed and the livers and brains removed for *in vitro* determination of their oxygen consumption. The liver was immediately placed in oxygenated phosphate buffered Ringer solution and sliced. The slices were transferred to Warburg flasks and the rate of oxygen consumption determined manometrically over a 2 hour period. The brain was removed as rapidly as possible and placed in oxygenated phosphate buffered Ringer solution without substrate. The cortex was separated, sliced, and the QO₂ of the cortical slices determined. With each determination, a simultaneous measurement was made of the QO₂ of liver and brain tissue from a normal rat of similar age and weight. The wet-dry weight ratio were determined separately in a series of liver slices from freshly killed rats, and the same factor was used throughout in calculating dry weights for the QO₂ values.

The liver slices from 9 of the experimental group gave an average QO₂ of 7.86 with a standard error of 0.23. This contrasts with the control value determined from 9 normal rats in which the average liver QO₂ was 6.42 with a standard error of 0.25. Thus the experimental QO₂ represents an increase of 22 per cent above the control level. The results are shown in table 1.

¹ The work described in this paper was in part carried out under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Cornell Medical College, and in part under a U. S. Public Health Service Research Grant.

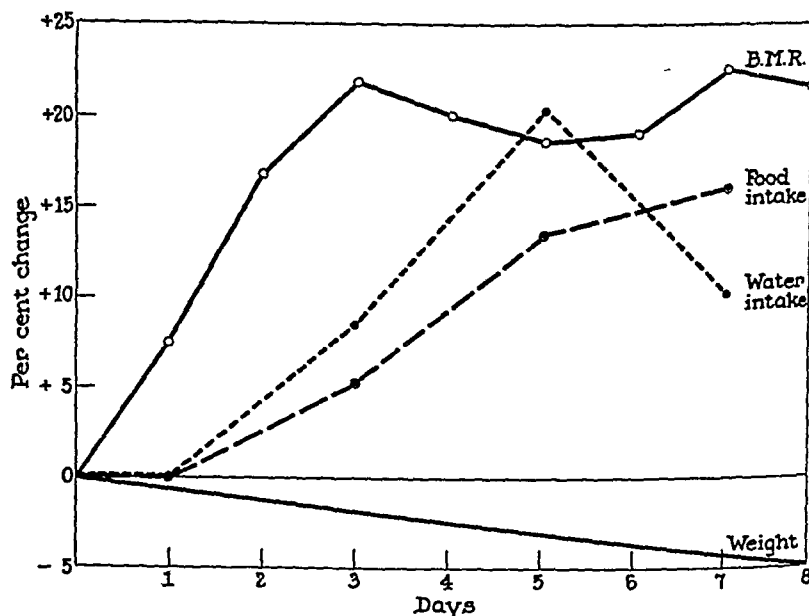


FIG. 1. CHANGES IN BASAL METABOLISM, FOOD INTAKE, WATER INTAKE, AND WEIGHT, IN RATS FED ON A DIET CONTAINING 0.1 PER CENT DDT

TABLE 1

*The effect of chronic DDT feeding on the oxygen consumption of the rat liver slice.
Experiment I*

RAT NO.	RAT WT.	NO. OF DAYS ON DDT DIET	SYMPTOMS	QO ₂	QO ₂ OF CONTROLS
	gm.				
1	212	8	4+	8.36 (3)*	6.30 (2)*
2	246	5	1+	7.33 (4)	6.73 (3)
3	215	9	4+	7.81 (3)	6.20 (3)
4	225	4	4+	7.17 (3)	6.25 (3)
5	194	9	4+	6.85 (2)	6.40 (3)
7	295	9	4+	7.95 (3)	5.38 (2)
8	219	8	1+	9.00 (4)	8.10 (1)
9	223	9	4+	7.75 (3)	5.80 (3)
12	265	7	4+	8.54 (4)	6.62 (3)
Average.....	233	8		7.86	6.42

* The figures in the parenthesis indicate the number of vessels.

The QO₂ of the slices of cerebral cortex from 9 experimental rats gave an average QO₂ of 7.56 with a standard error of 0.12. The cortical slices from the 9 control animals had an average QO₂ of 7.13 with a standard error of 0.12. This difference is not statistically significant. The results are shown in table 2.

To further evaluate the observed increase in the oxygen consumption of liver slices from DDT poisoned rats, the experiment was repeated on a second group of 16 rats similarly poisoned with 0.1 per cent DDT in the diet. The object of this experiment was to obtain the highest possible QO_2 values for liver from both the experimental group and the control animals in order to test the validity of the increased respiratory rate previously observed. This was accomplished by removing the liver as rapidly as possible and placing it in continuously oxygenated phosphate buffered Ringer solution maintained at $5^\circ C$. The tissue was sliced under these conditions and transferred to the same medium in Warburg flasks kept at this temperature until the vessels were attached to the manometers. The rate of oscillation of the vessels in the bath at $38^\circ C$. was increased from 100 per minute as in the first experiment to 130 per minute in the present experiment.

TABLE 2

The effect of chronic DDT feeding on the oxygen consumption of the rat brain cortex

RAT NO.	RAT WT.	NO OF DAYS ON DDT DIET	SYMPTOMS	QO_2	QO_2 OF CONTROLS
	gm.				
1	212	8	4+	7.62 (1)*	6.72 (3)*
2	246	5	1+	7.28 (3)	6.96 (3)
3	215	9	4+	7.68 (3)	7.10 (3)
4	225	4	4+	7.65 (2)	6.84 (3)
5	194	9	4+	6.82 (3)	7.06 (3)
7	295	9	4+	7.34 (3)	7.84 (3)
8	219	8	1+	8.16 (3)	7.14 (3)
9	223	9	4+	7.86 (3)	7.60 (3)
12	265	7	4+	7.70 (3)	6.94 (3)
Average	233	8		7.56	7.13

* The figures in parenthesis indicate the number of vessels.

Under these conditions the average QO_2 of liver slices from 15 control rats was found to be 7.81 with a standard error of 0.21. The average QO_2 of the liver slices from the DDT poisoned rats was 8.66 with a standard error of 0.15. The results are shown in table 3. It can be seen from this table that, with few exceptions, the QO_2 values of liver from the DDT poisoned rats is higher than that of the controls. The difference of approximately 11 per cent is smaller than was the case in the first series in table 1, but is statistically significant. It is probable that the higher oxygen utilization in the latter experiment was less favorable for a demonstration of the stimulant action of DDT. The results of both series, however, are in qualitative agreement. On the basis of these data it is concluded that liver tissue from DDT poisoned rats has a small but definitely increased respiratory rate.

To determine the effect of acute DDT intoxication on the liver QO_2 , 8 albino rats were given an intravenous injection of 50 mg. per kg. of DDT. The DDT was injected in a freshly prepared homogenized 2 per cent emulsion consisting of

torn oil, soya phosphatides, dibasic sodium phosphate, and water. When symptoms of poisoning were pronounced the animal was sacrificed, the liver immediately removed, placed in phosphate buffered Ringer solution continuously oxygenated at 5°C. and sliced.

The liver slices of these 8 rats gave an average QO_2 of 8.09. The average QO_2 of liver slices from 15 control rats (taken from table 3) was 7.91. The difference is insignificant. Thus the effect of DDT on liver respiration is not immediate.

It has been reported by others that the oxygen uptake of liver slices from rats chronically poisoned with DDT is either unchanged or decreased (2, 3). DDT fed to rats and other experimental animals produces varying degrees of liver

TABLE 3

*The effect of chronic DDT feeding on the oxygen consumption of the rat liver slice.
Experiment II*

RAT NO.	RAT WT.	NO. OF DAYS ON DDT DIET	SYMPTOMS	QO_2	QO_2 OF CONTROLS
	gm.				
1	165	19	4+	8.21 (5)*	7.04 (6)*
2	227	22	3+	8.57 (5)	7.50 (4)
3	229	22	3+	8.69 (6)	7.61 (5)
4	155	6	4+	8.69 (4)	8.13 (4)
5	154	6	4+	9.16 (4)	7.19 (4)
6	122	8	4+	10.22 (6)	7.18 (2)
7	156	6	4+	9.32 (5)	7.44 (6)
8	114	11	4+	8.66 (3)	8.88 (5)
9	174	8	4+	8.43 (4)	7.39 (5)
10	170	12	4+	8.55 (6)	7.21 (6)
11	210	20	1+	7.67 (8)	9.34 (3)
12	232	47	1+	7.86 (7)	7.90 (3)
13	165	5	2+	8.54 (6)	7.24 (3)
14	217	7	1+	8.54 (5)	8.88 (5)
15	175	8	2+	8.70 (6)	8.34 (4)
16	234	9	2+	8.12 (4)	—
Average . . .	181	14		8.66	7.81

* The figures in parenthesis indicate the number of vessels.

damage (4, 5). In view of this it is not surprising to find that the QO_2 of liver slices from chronically poisoned rats vary widely depending upon the portion of the liver selected for slicing. To test this point, liver slices from 9 animals weighing between 150 to 230 gm. were prepared from sections of the liver that appeared grossly altered after chronic DDT feeding. It was found that the QO_2 values of these slices averaged 6.61 and in some instances individual levels were as low as 5.00 (compare with control in table 3). It is not unlikely that the increase in oxygen consumption observed in liver slices from chronically poisoned rats was obscured to some extent by the presence of morphological damage.

To test the effects of DDT on the oxygen consumption of intact muscle, the following experiments were carried out on frogs. Normal healthy frogs were

injected with 50 mg. per kg. of DDT dissolved in corn oil. The injections were made into the ventral lymph sac. Twenty-four hours later 10 of 11 frogs manifested signs of toxicity. At this time they were pithed and the sartorii dissected out and placed in phosphate buffered Ringer's solution containing 1 mg./cc. of glucose, and the oxygen consumption measured manometrically. The QO_2 thus found was compared with that determined simultaneously on the sartorii of 11 normal frogs. The results are shown in table 4. The muscles from the poisoned frogs had an average QO_2 of 1.49 with a S.E. of 0.14 contrasted to a QO_2 of 1.22 for the control group with a S.E. of 0.14, a difference of 22 per cent.²

White and Sweeney reported the isolation of di(p-chloro-phenyl) acetic acid from the urine of rabbits fed DDT (6). They indicated that this represents an oxidation product of DDT formed in vivo by the rabbit. To determine whether or not this compound might be responsible for the characteristic DDT effects we

TABLE 4
The effect of DDT on the oxygen consumption of frog muscle

DDT POISONED FROGS				CONTROL FROGS		
Frog no.	Wt.	Symptoms of poisoning	QO_2 of sartorii	Frog no.	Wt.	QO_2 of sartorii
	gm.				gm.	
1	17	Present	1.49	1C	16	1.30
2	18	Present	1.41	2C	15	1.10
3	18	Present	1.67	3C	19	1.22
4	20	Present	1.10	4C	19	1.17
5	20	Absent	1.68	5C	25	0.90
6	22	Present	1.73	6C	23	1.36
11	14	Present	2.50	11C	26	2.25
12	15	Present	1.40	12C	21	0.85
13	15	Present	1.00	13C	14	1.00
14	19	Present	1.35	14C	24	1.50
15	22	Present	1.05	15C	19	0.80
Average	18.2		1.49		20	1.22

prepared and tested it on rat liver slices in vitro and also on the intact cat. A concentration of $1.8 \times 10^{-3}M$. had no effect upon the rate of oxygen uptake of normal rat liver slices. A cat was given a dose of 25 mg. per kg. intravenously as the sodium salt and 42 minutes later a dose of 50 mg. per kg. (approximately an LD 100, as DDT); no symptoms of poisoning were evident. These experiments indicate that this metabolite is not responsible for the characteristic manifestations of DDT poisoning.

The mechanism by which DDT increases the oxidative rate is not known, and experimentation in this direction is at present limited by the insolubility of the compound in aqueous media. Thus we have not succeeded in raising the oxygen

² The effect of DDT in increasing the oxygen consumption of frog muscle has been confirmed in a further series of experiments carried out in this laboratory by Mrs. Virginia Friend.

consumption by adding DDT to tissue *in vitro*. The extent to which the rise in metabolic rate may contribute to the characteristic neurological symptoms observed in poisoned animals is not established by these experiments. Against this possibility is the fact that we have been unable to demonstrate an increased metabolic activity of brain tissue from poisoned rats, and also the fact that in acute intoxication, the rat liver shows no significant change in the rate of oxygen uptake.

The effect of DDT upon metabolism resembles somewhat the pattern of action observed with the nitrophenols and the di- and tri- halophenols, as reported by Krah1 and Clowes (7). Further, it is noteworthy that compounds such as para-phenylenediamine and hydroquinone which affect the indolphenol oxidase system also simulate DDT in speed of lethal action and in the nature of symptoms produced in the intact animal (8).

SUMMARY

1. In rats poisoned with DDT there is an increase in the total metabolic rate.
2. The increased metabolic rate is reflected in an increase in food and water intake and a slight loss in body weight.
3. The oxygen uptake of liver slices from DDT poisoned rats is increased.
4. In advanced DDT poisoning, slices taken from parts of the liver showing gross abnormalities have instead of an increased oxygen uptake a reduction below the control values.
5. The oxygen consumption of sartorius muscles from frogs poisoned with DDT is increased.
6. A significant increase in the respiration of the brain tissue has not been demonstrated.
7. A known metabolite of DDT, di(p-chlorophenyl) acetic acid has been shown not to be responsible for the characteristic actions of DDT.

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EFFECT OF ORAL ADMINISTRATION OF DDT ON THE METABOLISM OF GLUCOSE AND PYRUVIC ACID IN RAT TISSUES

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The intoxication of mammals with DDT (2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane) results in tremors of varying severity and, if the dose is sufficiently high, in generalized tonic and clonic convulsions which may lead to respiratory failure and death (1, 2, 3). Although the symptoms appear to be of neurological origin,³ evidence has been submitted that, concomitant with their onset, there is an increase in body temperature and in the basal metabolic rate in rats.⁴ Accordingly, it was of interest to investigate whether metabolic changes in individual organs likely to be affected by DDT intoxication could be demonstrated.

EXPERIMENTAL. Methods. Adult rats, weighing 200 to 300 gms., were used in all experiments. The details of oral administration of DDT (m.p. 108.6–109.5°) are given in the tables. All animals had free access to food (Purina Dog Chow) and water at all times.

The rats were killed by a blow on the head. Cerebrum and cerebellum were homogenized separately in a Potter-Elvehjem glass homogenizer (4) and diluted with a medium containing 131 mM NaCl and 10 mM phosphate, pH 7.40, per liter, so as to contain about 30 mg. fresh weight of tissue per cc. of homogenate. Slices of liver, renal cortex and heart muscle were prepared free-hand and suspended in a medium containing 125 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂ and 10 mM phosphate, pH 7.40, per liter. In addition, these media contained either 0.1 per cent (5.5 mM) or 0.2 per cent (11 mM) glucose, or 0.055 per cent (5 mM) sodium pyruvate, prepared according to Robertson (5) and recrystallized from alcohol.

Aliquots of the homogenates were pipetted into Warburg vessels. The slices were dried quickly on filter paper, weighed on a torsion balance and transferred to Warburg vessels containing 3.0 cc. of medium in the main compartment and 0.2 cc. of 20 per cent KOH with filter paper in the center cup. The vessels and manometers were assembled and allowed to equilibrate with oxygen or purified nitrogen for about 10 minutes at 38° while shaking. After a further period of 3–4 minutes, replicate flasks were removed; 0.5 cc. aliquots from each flask (homogenate or medium) were pipetted into centrifuge tubes containing 4.5 cc. ice-cold 10 per cent trichloroacetic acid, mixed and centrifuged. In the supernatant fluid, the lactic acid content was determined by the method of Barker and Summerson (6), or the pyruvic acid content according to Friedemann and Haugen (7) (Extraction Method, Procedure B, with xylene as extractant).

Manometric readings were taken in those manometers where oxygen uptake was to be determined at intervals of 10 minutes for 60 minutes. At the end of this time, the remaining flasks were removed and their contents prepared for analysis as described for the zero time

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³Haymaker, W., and Ginzler, A. M., personal communication (1945).

⁴Cattell, McK., personal communication (1945).

samples. All determinations were carried out in duplicate. The results are expressed in the conventional way as Q values, *i.e.*, c mm. of change per hour per mg. initial dry weight of tissue. Dry weights were determined by drying aliquots of homogenates, or representative slices, at 105° for 24 hours and Q values so obtained were about 20 to 25 per cent lower than those obtained when the final dry weights of tissue slices were determined.

In all series of experiments, one half of each group of control and experimental rats was male. Evaluation of the results to be presented showed no sex difference, and the results of male and female rats were therefore combined.

Effect of prolonged DDT intoxication. On a daily single dose of 50 mg. DDT per kgm. of body weight (2.0 cc. of a 2.5 per cent solution in corn oil) by stomach

TABLE 1

Effect of chronic DDT intoxication of rats on tissue respiration, aerobic and anaerobic lactic acid production

Experimental rats received daily by stomach tube 2.0 cc. of 2.5 per cent DDT in corn oil (50 mg. DDT) per kg. of body weight, control rats 2.0 cc. of corn oil per kg., for 30 to 50 days. All results are averages of duplicate determinations on 10 control rats or 10 DDT-intoxicated rats. Substrate: 0.1 per cent glucose. Results are expressed as Q = c.mm. gas produced per hour per mg. initial dry weight.

	CONTROL RATS	DDT RATS	SIGNIFICANCE OF DIFFERENCE P
Q_{O_2}			
Cerebral homogenate	-7.32	-6.94	0.4
Cerebellar homogenate	-4.62	-5.55	0.15
Liver slices	-6.03	-6.53	0.06
$Q_{LA}^{O_2}$			
Cerebral homogenate	+2.45	+2.20	0.5
Cerebellar homogenate	+1.48	+1.52	0.9
Liver slices	+0.78	+0.50	0.02
$Q_{LA}^{N_2}$			
Cerebral homogenate	+6.27	+5.86	0.3
Cerebellar homogenate	+4.92	+6.07	0.2
Liver slices	+2.05	+1.16	0.01

tube, all rats developed moderate to marked tremors within 4 hours after administration, and some of them were prostrate. These symptoms lasted for at least 5 hours, but had uniformly disappeared at the end of 18 hours after administration, at which time animals were used for metabolic study.

From the results presented in table 1 it may be seen that such treatment for 30 to 50 days resulted in no significant effect on the oxygen uptake, aerobic and anaerobic lactic acid production of cerebral and cerebellar homogenates. There was also no significant effect on the oxygen consumption of liver slices, but a significant decrease in their aerobic and anaerobic production of lactic acid.

Table 2 shows that prolonged poisoning with DDT for 70 to 100 days did

TABLE 2

Effect of chronic DDT intoxication on tissue respiration in presence of pyruvic acid and on utilization of pyruvic acid

Experimental rats received daily by stomach tube 2.0 cc. of 2.5 per cent DDT in corn oil (50 mg. DDT) per kg. of body weight, control rats 2.0 cc. of corn oil per kg., for 70 to 100 days. Substrate: 0.055 per cent sodium pyruvate. Results are expressed as $Q =$ c.mm. gas produced per hour per mg. initial dry weight.

	CONTROL RATS (6)*	DDT RATS (9)*	SIGNIFICANCE OF DIFFERENCE <i>P</i>
Q_{O_2}			
Cerebral homogenate.....	-6.0	-6.5 (8)	0.1
Cerebellar homogenate.....	-4.0 (5)	-4.4	0.4
Liver slices.....	-7.3	-7.7	0.3
Kidney cortex slices.....	-18.3	-20.8	0.04
Heart slices.....	-8.6	-10.3	0.1
$Q_{pyv.}$			
Cerebral homogenate.....	-4.6	-4.7 (8)	0.6
Cerebellar homogenate.....	-3.3	-3.8	0.1
Liver slices.....	-5.2	-5.7	0.2
Kidney cortex slices.....	-11.7	-12.7	0.4
Heart slices....	-4.9	-4.8	0.9

* Unless indicated otherwise by figures in parentheses.

TABLE 3

Effect of acute DDT intoxication of rats on liver slice respiration

Experimental rats received DDT as 0.2 per cent of their diet. Substrate: 0.2 per cent glucose. Results are expressed as $Q =$ c.mm. gas produced per hour per mg. initial dry weight.

Average Q_{O_2} of control rats (20) = -6.64 (S.D. = 0.76)

LENGTH OF TREATMENT	Q_{O_2}	DIFFERENCE FROM AV. OF CONTROLS	SYMPTOMS (TREMORS)	LENGTH OF TREATMENT	Q_{O_2}	DIFFERENCE FROM AV. OF CONTROLS	SYMPTOMS (TREMORS)
<i>days</i>		<i>per cent</i>		<i>days</i>		<i>per cent</i>	
1	-6.80	+2	none	5	-8.45	+27	moderate
1	-7.45	+12	slight	5	-7.55	+14	severe
2	-8.20	+24	none	6	-7.40	+11	severe
2	-7.40	+11	slight	6	-8.50	+28	severe
3	-8.05	+21	moderate	7	-6.35	-4	moderate
3	-7.10	+7	moderate	7	-6.35	-4	severe
4	-8.15	+23	severe	8	-7.05	+6	moderate
4	-8.95	+35	moderate	9	-7.10	+7	severe
				10	-7.20	+9	severe

Average Q_{O_2} of experimental rats (17) = -7.53 (S.D. = 0.75).

Difference from average of controls = +13 per cent.

$t = 3.59$; $P = < 0.01$.

not produce any significant change in oxygen uptake in the presence of pyruvate, or in the rate of disappearance of pyruvic acid, in cerebral and cerebellar homogenates or in slices of liver, renal cortex and heart.

Liver slice respiration in rats receiving a diet incorporating DDT, and sacrificed after 1 to 10 days. Since other laboratories^{3,4} had reported conflicting changes in the oxygen consumption of liver slices from rats which had been fed DDT in their diet, experiments were carried out on rats which received a diet incorporating 0.2 per cent DDT and which showed symptoms of intoxication at the time of sacrifice. Table 3 shows that there is an increase in liver slice respiration of DDT poisoned animals which is small (13 per cent) but definitely significant ($t = 3.59$; $P < 0.01$).

DISCUSSION. The administration of DDT to rats for one to 10 days increases the respiration of the livers from these animals significantly above that of livers from control rats. These results confirm the findings of Cattell *et al.*⁴ Barron and Hill⁵ have found that, in the absence of added substrate during the manometric determination but with an otherwise similar experimental arrangement, there was a decrease in the oxygen consumption of liver slices, to 45 per cent of normal values, from animals exposed to a diet containing 0.2 per cent DDT for 1 to 8 days. In view of Barron's⁵ findings that during the acute stage of DDT intoxication, the liver stores of glycogen are depleted, it is possible that this decrease in the respiratory rate was due to the absence or unavailability of the substrate within the tissue cells, while in the present experiments and in those of Cattell, such substrate was supplied *in vitro*. On the other hand, it is of interest to note that daily intoxication of rats with DDT over a period of from 30 to 100 days did not materially affect the ability of the tissues studied to utilize glucose or pyruvic acid, in spite of the fact that these animals showed daily evidence of poisoning. This lack of metabolic disturbance may have been due either to the fact that the rats had no symptoms at the time of sacrifice, or that the dose used may have been lower than that ingested in the diet.

The authors wish to thank Miss Priscilla Day for valuable technical aid in carrying out these studies.

SUMMARY

1. Daily administration of 50 mg. DDT per kg. in corn oil to rats by stomach tube for 30–50 days produced, at a time when the animals were free from symptoms of acute intoxication, no change in the oxygen consumption, aerobic and anaerobic lactic acid production of cerebral and cerebellar homogenates and in the oxygen consumption of liver slices, in the presence of added glucose. Aerobic and anaerobic lactic acid production in liver slices was depressed to a small but statistically significant degree.

2. Rats treated as before for 70–100 days showed no significant change in the respiration rate with pyruvic acid as substrate, or in the utilization of pyruvic

³Barron, E. S. G., and Hill, D. L., personal communication (1945).

⁴Barron, E. S. G., personal communication (1945).

acid, by cerebral and cerebellar homogenates and by liver, kidney cortex and heart slices.

3. When DDT was administered to rats by incorporation in their diet for periods of 1-10 days, liver slices from such rats showed significant increase in the oxygen consumption above that of control animals.

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OBSERVATIONS ON THE EFFECTS OF TRIMETHYL PHOSPHATE UPON EXPERIMENTAL ANIMALS

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It is believed that information on the toxicity of trimethyl phosphate and on the signs of intoxication shown by animals poisoned by the compound, will be of interest because of the chemical relationship of this compound to tri-cresyl phosphate and other closely related materials. Some present practical usefulness of this information may also arise from the fact that trimethyl phosphate has been found to have commercial applications.

The scope of these observations was concerned with the response of experimental animals to the oral and subcutaneous administration of trimethyl phosphate and to its application upon the skin.

EXPERIMENTAL PROCEDURES. *Choice of animals.* Albino rabbits and rats, guinea pigs, and a cat were employed for these observations. The rats were reared in the laboratory from Sprague-Dawley stock. All other animals were purchased from a local supplier.

Oral administration. The compound was administered to rabbits by means of a rubber stomach tube and to guinea pigs and rats by means of a blunt hypodermic needle traversing the esophagus. The principles determining the selection of choice of the dosages administered have been discussed elsewhere (1). In the case of rabbits and rats, the toxicity is expressed in terms of LD_{50} as calculated from the experimental data by the method of maximum likelihood of Bliss (2), but in the case of guinea pigs, fewer animals were used and only the approximate lethal dose was determined.

Cutaneous application. The hair over the abdominal wall of a rabbit was clipped closely. One dose of 2.0 ml. of trimethyl phosphate per kilogram of body weight was applied on each of five days per week, wetting an area of about 200 sq. cm. on each occasion. After two or three hours, the compound was removed by washing with soap and water. The total number of applications upon the skin of individual animals ranged from 5 to 20.

Metabolic observations. Twenty-four hour specimens of the urine of rabbits given trimethyl phosphate were analyzed for protein, reducing substances, hexuronic acid (3), and for organic and total sulfates (4).

RESULTS. *Physiologic effects of the absorption of trimethyl phosphate.* When absorbed in a lethal concentration from the gastroenteric tract of a rabbit, guinea pig or rat trimethyl phosphate induced a gradually decreasing rate and amplitude of respiratory movements (sometimes after a brief period of stimulation), general weakness, mild hyperirritability, and fine tremors. These signs were followed by marked dyspnea, collapse and death by respiratory failure. The blood pressure of rabbits (anesthetized by sodium barbital and given a lethal oral dose of trimethyl phosphate) decreased steadily but the heart was seen beating for several minutes after respiration had ceased.

The repeated administration of sublethal doses to rabbits resulted in similar signs of poisoning and also in the frequent occurrence of loss of body weight and

of flaccid paralysis of the extremities, which in some instances were superseded by a spastic state of the effected muscles.

The immediate toxicity of trimethyl phosphate when administered orally. The lethal oral dose of trimethyl phosphate, administered at one time to the rabbit,

TABLE 1

Immediate toxicity of trimethyl phosphate when administered in a single oral dose to rabbits and rats

NUMBER OF ANIMALS USED	DOSE	PERCENTAGE OF DEATHS	SURVIVAL TIME	LD ₅₀
Rabbits				
6	0.62	0		1.05 ml /kg.
10	0.94	20	5 and 7 days	
10	1.2	80	30 hrs. to 5 days	
10	1.4	100	30 to 48 hrs.	
6	2.1	83	24 to 35 hrs.	
6	3.2	100	24 to 36 hrs.	
6	4.7	100	5 to 24 hrs.	
Rats				
10	0.94	0		1.65 ml./kg.
10	1.4	50	2 to 8 days	
10	1.75	30	30 hrs. to 7 days	
10	2.1	100	24 hrs. to 8 days	
10	3.2	100	20 hrs. to 3 days	
10	4.7	100	15 hrs. to 3 days	

TABLE 2

Immediate toxicity of trimethyl phosphate when administered in a single oral dose to guinea pigs

NUMBER OF ANIMALS USED	DOSE	FATE OF ANIMAL	APPROXIMATE LETHAL DOSE
	ml./kg.		
2	0.42	survived	1.4 ml./kg.
2	0.62	survived	
2	0.94	one died in 30 hrs.	
2	1.4	died in 24 and 30 hrs.	
2	2.1	died in 10 hrs. and 3 days	
2	3.2	died in 7 and 16 hrs.	
2	4.7	died in 7 and 8 hrs.	

rat and guinea pig, is 1.05 ml. (LD₅₀), 1.65 ml. (LD₅₀), and 0.94 ml. (approximate), respectively, per kilogram of body weight (tables 1 and 2).

Effects resulting from repeated cutaneous applications of trimethyl phosphate. Trimethyl phosphate was applied upon the skin of each of six rabbits in a dose

of 2 ml. per kilogram of body weight, for two hours per day on each of 20 days over a total period of 28 days. No signs of local cutaneous irritation were observed and all animals survived. That the compound is capable of passing through the abdominal skin in sufficient quantity to induce poisoning is suggested by the loss of body weight which occurred in half of the animals, and by the development, in one instance, of flaccid paralysis following the last application. Three days later the paralysis gave way to a state which, in certain respects, resembled that induced by the absorption of tri-*o*-cresyl phosphate. This rabbit assumed a hunch-backed position which may have resulted from the contraction of the psoas muscle and the relaxation of the sacrospinal muscles. The posture of the rabbit given trimethyl phosphate differed however from that associated with poisoning by tri-*o*-cresyl phosphate, in that the forelegs, and hindlegs from knees to toes, were rigidly extended; the hip joint was flexed.

To exclude the possibility that these manifestations may have resulted from a cause unrelated to the absorption of trimethyl phosphate, the experiment was repeated upon three other rabbits in a corresponding manner, except that each period of contact with the compound was increased to three hours. One rabbit died after 5 applications, and another after 14, these animals having lost 546 and 1213 g. of body weight, respectively. Both animals exhibited fine tremors and unsteadiness, together with weakness and incoördination of the lower extremities. After 3 or 4 applications, the third rabbit, which lost only 13 g. of body weight, was observed to be lying with its legs extended. When touched it would assume a normal sitting position, or hop about, then exhibiting fine tremors and unsteadiness. Paralysis of the muscles of the extremities developed after the seventh application, and was followed, as in the instance referred to previously, by spasticity. The rabbit was killed after the 14th application.

Effects resulting from repeated oral and subcutaneous administration of trimethyl phosphate. Trimethyl phosphate was administered repeatedly, by a stomach tube to rabbits and subcutaneously to a cat, in an effort to determine whether the neuromuscular disturbances described above could be brought about by the alimentary or subcutaneous absorption of the compound.

Each of three rabbits was given an oral dose amounting to 0.3 ml. of trimethyl phosphate per kilogram of body weight, on each of 6 succeeding days. The total dose administered to each animal was equivalent to nearly twice the single lethal dose. One animal gained 303 g. in body weight, while the other two lost 135 and 418 g., respectively. All of these animals developed fine tremors, unsteadiness and weakness of the extremities, after the second or third dose, and in each instance, a flaccid paralysis developed two days later. A few days after the last dose had been administered the initial flaccid paralysis gave way to a state of spasticity.

A cat was given 79 subcutaneous injections of trimethyl phosphate (each equal to 0.1 ml./kg.) over a total period of 123 days. General weakness became apparent after administration of 30 doses. This impairment increased somewhat in severity as the injections continued, but no paralysis developed. The animal lost 600 g. in body weight.

Examination of urine of rabbits given trimethyl phosphate. A twenty-four hour specimen of urine was collected from each of 6 rabbits given one oral dose of about 1 g. of trimethyl phosphate per kilogram of body weight. Neither proteins nor reducing substances were found in any specimen. The values for the hexuronic acid content of the urine remained within the normal range (14 to 51 mg.), but the ratio of organic to total sulfates in the urine of the individual animals increased to 13, 14, 24, 27, 32 and 41 per cent, respectively (normal ratio about 10). This observation indicates that some portion of the absorbed trimethyl phosphate, or some product of its metabolism, is excreted as an organic sulfate.

DISCUSSION. The signs of intoxication observed following the absorption of trimethyl phosphate by animals, are of interest because they resemble those associated with the absorption of certain other phosphoric and phosphorous acid esters. For instance, tri-*o*-cresyl phosphate, like trimethyl phosphate, causes hyperexcitability, fine tremors, incoördination and muscular weakness, as well as paralysis and spasticity of certain groups of skeletal muscles, death occurring as a result of respiratory failure. The effects induced by the absorption of other closely related compounds have been reviewed with respect to the neurotoxic manifestations. From the available evidence it is not possible to find any logical correlation between such manifestations and the chemical structure of these compounds. In addition to tri-*o*-cresyl phosphate (5, 6, 7, 8), the following materials appear to have neurotoxic effects: tri-*o*-cresyl thiophosphate (6), triphenyl phosphate (6, 9), triphenyl phosphite (10), tri-*o*-cresyl phosphite (6, 10), tri-*m*-cresyl phosphite (10), tri-*p*-cresyl phosphite (10), and catechol phosphate (6). Until proof to the contrary is available, these compounds, in addition to trimethyl phosphate, may well be considered potentially capable of inducing neuritis or related disorders in man, a fact which has been well established in the case of tri-*o*-cresyl phosphate (12 to 33). Absorption of the following other closely related compounds seems not to have induced neurotoxic manifestations in animals: tri-*m*-cresyl phosphate (6), tri-*p*-cresyl phosphate (6), monosodium di-*o*-cresyl phosphate (11), disodium mono-*o*-cresyl phosphate (11), triphenyl thiophosphate (6), and guaiacol phosphate (6, figs. 6 and 7).

CONCLUSIONS

1. When absorbed from the gastroenteric tract of rabbits, rats or guinea pigs in sufficient quantity over periods of a few hours or days, trimethyl phosphate induced an acute toxic state characterized by a gradual decrease in the rate and magnitude of respiratory movements, general body weakness, a mild degree of hyperexcitability, fine tremors, dyspnea, collapse and death as a result of respiratory failure. When sublethal oral, cutaneous or subcutaneous doses were administered day by day for several days to rabbits a corresponding type of intoxication developed more slowly and was accompanied in some instances by loss of weight and flaccid paralyses of the extremities, the latter being followed at times by spasticity of certain muscles of the extremities.

2. The lethal oral dosage of trimethyl phosphate for the rabbit is 1.05 ml./kg., for the rat, 1.65 ml./kg., and for the guinea pig, 0.94 ml./kg.

3. Some portion of the administered trimethyl phosphate or some product of its metabolism appears to be excreted in the urine of rabbits in conjugation with sulfuric acid.

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ON THE ANTICONVULSANT ACTIVITY OF VARIOUS NITROGEN HETEROCYCLES: I. ALKYLTHIOETHERBARBITURATES AND II. PHENYLALKYLTHIOMETHYLHYDANTOINS

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The technique of Putnam and Merritt (1, 2), has proved to be of such value that its use led to the discovery of new antiepileptic drugs (2, 3, 4). No complete and systematic study, however, has yet been made for the purpose of finding the relationship between structural features and antiepileptic activity. The technique of Putnam and Merritt, after slight modifications, has been applied to 55 new compounds belonging to two different types of nitrogen heterocycles: alkylthioether derivatives of barbituric or thiobarbituric acid, and phenylalkylthiomethylhydantoin derivatives.

For the selection of anticonvulsants promising for clinical experimentation, three different factors have been separately taken into consideration: 1) intensity of anticonvulsant activity measured by different techniques in different animal species; 2) duration of anticonvulsant activity; 3) toxicity and absence of side effects (ataxia and narcosis).

A. EXPERIMENTAL. Technique. The technique of Putnam and Merritt was followed as described in Science (1). However, instead of a rectangular current, a sine-wave current was used for the production of convulsive seizure. The apparatus, similar to the stimulator described by Tainter (5), consists essentially of a Variac transformer employed on an A.C. 60 cycle current line which passes through a relay to the electrodes. Three modifications, however, increase the consistency of the technique: (1) use of an interval timer which automatically shuts off the current and avoids the errors due to subjective methods of timing, (2) use of clamps, clipped directly on the occipital skin and the ear, in order to avoid the excitation of the animal and waste of time in strapping the electrode to the occipital hair and installment of a bit in the mouth, (3) use of "Electrode Jelly" on the skin and ear for improving conductivity instead of moistening the hair.

Experimental conditions. The drug was administered to 12-hour fasting cats by the oral route, either in solution or in suspension with acacia. At least 6 animals were used for each dosage level.

Determination of the threshold. The minimal amperage of current necessary to produce convulsions in the cat after 10 seconds electrical shock was considered as an index of the convulsive threshold. Threshold value was established before and 90 minutes after the administration of the drug. As recommended by Merritt et al (2) stimulations in excess of 50 mA were never given; therefore, a small dose of drug was always used as selected from preliminary experiments.

Calculation of the anticonvulsant activity. The anticonvulsant activity of a substance was rated by the increase produced in the threshold of electroshock seizure. For comparative study of antiepileptic drugs, the threshold increase was calculated at the peak of action as an average of at least 6 cats. Activity was measured in comparison with phenobarbital and dilantin, given to the same animals at weekly intervals.

Duration of anticonvulsant action. The duration of anticonvulsant action was studied

by measuring the threshold of electrical convulsions in the same animal at intervals of 2 hours, during a period ranging between 2 and 24 hours. In controls, the determination of the threshold, repeated at intervals of at least 90 minutes, showed that it remained surprisingly constant (10% variation) in the same day; this confirms previous observations (2, 18).

Detection of side effects. After medication the animal was placed in an observation cage, and before electrical stimulation, tested for central impairment. Hypnosis was measured by the loss of righting reflex. Ataxia was studied according to a standardized procedure: observation of spontaneous locomotion and of turning a partial somersault during a jump from a two-foot high platform.

Antagonism against metrazol. In addition to protection against electroshock, anticonvulsant action against metrazol was used for certain drugs. A technique recently described (6, 7) consists of measuring the protective effect against convulsions induced by subcutaneous injection of metrazol in the rat. A nontoxic dosage level of 50 mgm./kgm. was used rather than 75 mgm./kgm. as used by the previous authors. The percentage of metrazol convulsions in subjects receiving the drug before metrazol was noted in comparison with controls receiving metrazol alone. At least 24 rats were used for each dose of drug in comparison with 24 controls with each experiment. Duration of antiepileptic action was measured by the duration of protection against metrazol.

Acute intravenous toxicity. The mean lethal dose (LD 50) was determined in albino male rats (weight 100-225 grams) using slow intravenous injection (0.1 cc. of a 2% solution in 15 sec.).

B. MATERIAL. *Alkylthioetherbarbiturates and alkylthioetherthiobarbiturates.* 45 alkylthioetherbarbiturates and thiobarbiturates listed in table 1 were investigated.¹ In these compounds, X represents either oxygen (alkylthioetherbarbiturate) or sulphur (alkylthioetherthiobarbiturate). R and R1 represents various alkyl substituents (methyl, ethyl, propyl, isopropyl, butyl, isobutyl, amyl, isoamyl, etc.)

Phenylalkylthiomethylhydantoin derivatives. Table 1 concerning also ten homologues of Diphenylhydantoin where the alkyl group is represented by one of the following: ethyl, normal propyl, isopropyl, butyl and amyl.²

C. RESULTS. *Anticonvulsant activity in the cat.* The results presented in tables 2 and 3 summarize the percentage of increase in threshold, duration of anticonvulsant action, and observation of side effects of alkylthioether derivatives at the standard dosage level of 5 mgm./kgm. and 10 mgm./kgm. orally. In addition, the LD 50 (intravenous rat) is presented in the tables.

It is apparent that nine compounds: Nos. 60, 68, 192, 100, 92, 199, 114, 119, and 211, have about the same anticonvulsant activity as phenobarbital and diphenylhydantoin-sodium. A clear-cut difference between these thioether derivatives and the reference compounds is that with the exception of Nos. 60, 68, 100, 119, and 211, ataxia is induced at the effective dose level. Another distinctive feature lies in the shorter duration of action (4-6 hours for most of them and 16 hours for #100) instead of the 24 hours observed with phenobarbital and diphenylhydantoin-sodium.

Results summarized in table 4 show the corresponding doses of phenylalkylthiomethylhydantoin derivatives having the same anticonvulsant activity (33%

¹These compounds have been prepared by Dr. L. A. Walter and Dr. W. Goodson of the Chemical Department.

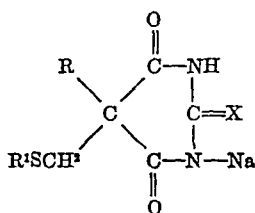
²These compounds have been prepared by Dr. R. Barry, Dr. L. A. Walter, and Dr. A. Popkin of the Chemical Department.

TABLE 1

Structure of various compounds studied

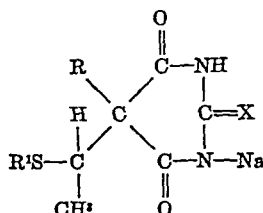
I. ALKYLTHIOMETHYL DERIVATIVES

1. Alkylthiomethyl derivatives



2. Alkylthioalkyl derivatives

a. Alkylthioethyl



NO.	SUBSTITUTION		X	
	R	R ¹		
Barbiturates				
60	Propyl	Ethyl	O	
59	Isopropyl	Ethyl		
62	Sec. butyl	Ethyl		
68	Methyl-butyl	Ethyl		
58	Isoamyl	Ethyl		
Thiobarbiturates				
57	Ethyl	Ethyl	S	
81	Isobutyl	Ethyl		
83	Sec. butyl	Ethyl		
107	Cyclohexyl	Ethyl	O	
Barbiturates				
55	Allyl	Ethyl		
63	Methyl	Ethyl		
85	Isobutyl	Methyl		
192	Methyl-butyl	Methyl		
88	Allyl	Propyl	S	
94	Allyl	Butyl		
164	Methyl-butyl	Allyl		
91	Butyl	Butyl		
Thiobarbiturates				
86	Isobutyl	Methyl	O	
Barbiturates				
56	Ethyl	Ethyl		
74	Ethyl	Isopropyl		
105	Ethyl	Allyl		
100	Ethyl	Ter. Butyl	S	
92	Ethyl	Amyl		
104	Ethyl	Methyl. but.		
117	Ethyl	Ethyl. but.		
Sulfone				
53	n-butyl	Sulfone		

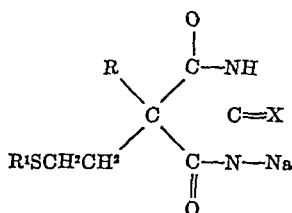
CH ²		O	
Barbiturates			
138	Propyl	Methyl	O
165	Ethyl	Ethyl	
199	Allyl	Ethyl	
200	Allyl	Allyl	
125	Isobutyl	Ethyl	
114	Allyl	Butyl	S
109	Ethyl	Butyl	
115	Ethyl	Amyl	
Thiobarbiturates			
166	Ethyl	Ethyl	
124	Ethyl	Butyl	S
128	Butyl	Amyl	

b. Alkylthiopropyl

NO.	SUBSTITUTION			
	R	R ¹	R ²	X
Barbiturates				
119	Ethyl	Butyl	Ethyl	O
Thiobarbiturates				
122	Ethyl	Butyl	Ethyl	S

TABLE 1—Continued

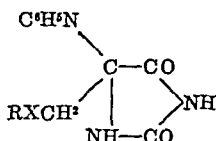
3. Alkylthioethyl derivatives



NO.	SUBSTITUTION		
	R	R ¹	X
Barbiturates			O
116	Ethyl	n-butyl	
168	Allyl	Ethyl	
211	Allyl	Isopropyl	
155	Allyl	n-butyl	S
Thiobarbiturates			
151	Ethyl	n-butyl	
152	Allyl	Butyl	

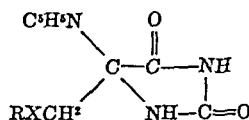
II. PHENYLALKYL/THIOMETHYL-HYDANTOIN DERIVATIVES

1. Phenylalkylthiomethyl-hydantoins



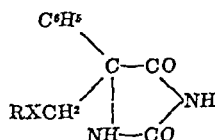
No.	SUBSTITUTION	
	X	R
228	S	Methyl
218		Ethyl
229		n-Propyl
230		Isopropyl
220		n-Butyl
221		n-Amyl

2. Pyridylalkylthiomethyl-hydantoins



232	S	
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3. Alkylsulfonemethyl-hydantoins



219	SO ²	Ethyl
225		n-Butyl
226		n-Amyl

increase in the electroshock convulsive threshold). Diphenylhydantoin-sodium^{*} has been used as a reference compound at the dose of 5 mgm./kgm. orally. None of these compounds is as potent as diphenylhydantoin-sodium. The most promising of the phenylalkylthiomethylhydantoins, #218, is only one-fourth as active as diphenylhydantoin sodium. The duration of its action is approximately the same (20 hours). Also, it may be noted in table 4 that the ratio of antiepileptic dose/ataxic dose remains the same for all these compounds. None of the alkylsulfonemethylhydantoins is active under 100 mgm./kgm. which induce ataxia in the cat.

Anticonvulsant activity in the rat. The activity of some phenylalkylthiomethylhydantoins prompted the examination of their protective activity against metrazol convulsions in the rat.

^{*}This drug has been made available by the courtesy of Dr. L. A. Sweet of Parke, Davis and Co.

As presented in table 5, diphenylhydantoin-sodium (administered orally) is unable to protect against metrazol convulsions if given during either one, two, or three days at the dosage level of 60 mgm./kgm. each day (30 mgm./kgm. twice a day). After $4\frac{1}{2}$ days, and with a total administration of 270 mgm./kgm., protection occurs; the percentage of protection against convulsions induced by 50

TABLE 2
Anticonvulsant effect of alkylthioether derivatives

REFERENCE NO.	LD ₅₀ RAT I.V.	DOSE OF 10 MG./KG.		DOSE OF 5 MG./KG.		DURATION OF ACTION (10 MG.)	SIDE EFFECTS, EFFEC. DOSE	ATAXIC DOSE
		Inc. after 1½ hr.	Inc. peak of action	Inc. after 1½ hr.	Inc. peak of action			
I. Alkylthiomethylbarbiturates and thiobarbiturates								
	mgm./kgm.	%	%	%	%	hours		
60	—	28.3	28.3	16.5	16.5	4	0	10
59	—	20	20	0	0	2	A	—
62	170	38	38	0	0	3	A	—
68	120	34	43	16.1	16.1	24	0	—
58	130	48.9	62	0	0	7	A	—
57	—	10	10	0	0	5	0	—
81	—	20	20	0	0	6	0	—
83	260	24.6	24.6	0	0	4-7	S	—
107	—	26	26	15	15	5-7	N	20
55	—	6	6	0	0§	2	A	10
63	—	0	0	0	0	0	0	—
85	170	28	28	18	18	4	A	10
192	100	54.5	60	17.4	20.7	4-8	A	—
88	—	17.3	33	11.2	11.2	—	D	—
94	100	0	0	0	0*	—	A	—
164	110	48.7	58.7	15	15	3½	D or A	—
91	90	22.5	22.5	14.1	8.5	4	D	—
86	—	15	15	0	0	4	0	—
56	—	15	15	0	0†	4	0	—
74	—	0	0	0	0	0	0	—
105	100	10	23	0	0	6-24	0	—
100	—	26	26	25	25	6-16	0	15
92	—	24	58.6	22.8	22.8	6	A	—
104	100	16	20	18.5	20	6	A	—
117	130	10	10	0	0‡	2	D	—

* At the dose of 20 mgm: 27%.

† At the dose of 20 mgm: 12%.

‡ At the dose of 20 mgm: 43%.

§ At the dose of 20 mgm: 17%.

A = ataxic; S = sluggish; D = down; 0 = none.

mgm./kgm. of metrazol increases 30%. The same observations can be made with *218, *220, *229, and *230. The examination of table 5 shows also that on subcutaneous administration, diphenylhydantoin-sodium is inactive even at the dose of 150 mgm./kgm. In table 6, ratios of the comparative activity of various alkylthiomethylhydantoins appear to be quite the same whether electrical shock in the cat or metrazol convulsions in the rat is used as the technique.

DISCUSSION. The above study was limited to screening pharmacological tests made in order to find a drug promising for clinical assay in "grand mal" and psychomotor seizures.

TABLE 3
Anticonvulsant effect of alkylthioether derivatives

NO.	LD 50 RAT I.V.	DOSE OF 10 MG./KGM.		DOSE OF 5 MG./KGM.		DURATION ACTION (10 MG.)	SIDE EFFECTS, EFFEC. DOSE	ATAXIC DOSE
		Inc. after 1½ hr.	Inc. peak of action	Inc. after 1½ hr.	Inc. peak of action			
II. Alkylthioalkylbarbiturates and thiobarbiturates								
(a) Alkylthioethyl								
	mgm./kgm.	%	%	%	%	hours		mgm./kgm.
138		33	33	30	30	5	A	15
165	160	0	12	0	8	4	A & W	
199	120	—	37.6	—	20	2	A	
200	95	35	35	0	0	6	A	
125	110	20	20	9.5	9.5	5-6	A or D	
114	90	51	77	30	30	4	D	
109	90	30	38.5	17.6	17.6	5	A or D	
115	90	0	0	0	0	—	A	
166	100	40.9	40.9	12	12	5-4	A	
124	90	10.8	10.8	—	—	6	A or D	
128	110	10	—	—	—	2	A or D	
(b) Alkylthiopropyl								
119	—	35	49.7	26.9	26.9	3-6	0	
122	70	10	10	0	0*	3	A	
III. Beta-alkylthioethylbarbiturates and thiobarbiturates								
116	130	30	48	4	5	7	A	10
168	—	66	66	40	40	4.5	A	
211	—	100	100	45	45	3.5	0	
155	80	20	20	0	0	3.5	A	
151	140	15.4	15.4	0	0	4	A	
152	120	5	5	—	†	2	0	
Reference compounds								
Phenobarbital.....	14.3	56.3	10.6	37.5	24	0		
Diphenylhydantoin sodium.....	20.6	53.5	8.6	33	28-29	0		

* At the dose of 20 mgm./kgm.—45%.

† At the dose of 20 mgm./kgm.—56%.

A = ataxia; W = wobbly; D = down; 0 = none.

(1) *Method of assay.* The technique of Putnam and Merritt involving the protection against electroshock seizures in cats has been found to be very reliable. The criterion used for the determination of the seizure threshold concerns the *tonic-clonic motor sequence posterior to the stimulation*, which is a *continuing after-effect* of the electrical stimulation of the motor cortex and has for a long time been

TABLE 4
Anticonvulsant effect of phenylalkylthiomethylhydantoin derivatives

REFERENCE NO.	M.P.	EFFECTIVE DOSE		DURATION OF ACTION	SIDE EFFECTS AT EFFECTIVE DOSE	ATAXIC DOSE 100%
		Average 33% rse. thresh.	Minimum 15% rse. thresh.			
	°C.	mgm./kgm.	mgm./kgm.	hrs.		mgm./kgm.
Dilantin	292	5	—	26	0	25-30
Phenyl-alkylthiomethylhydantoin						
228	162.5-163	50	30	3-20	A-3 hrs.	50
218	186.5-188	20	5	20	0	40
229	139.5-140	30	20	6	Sl. A-1 hr.	50
230	151-154	30-50	25	6	A-3 hrs.	50
220	107.5-108.5	40	10	5-20	A-3 hrs.	40
221	94-95	60	20	20	A-3 hrs.	50-100
Pyridylthiomethylhydantoin						
232	234-235	30	20	5	A. death aft. 2 das.	40
Phenyl-alkylsulfomethylhydantoin						
219	229-229.5	100	50	22	0	50
225	190.5-191	200	50	22	D	
226	181-182	100	—	20	A	

A = ataxia; Sl. = slight; 0 = none; D = down.

TABLE 5
Comparative percentage of protection against metrazol convulsions induced by phenylalkylthiomethylhydantoins

REFERENCE NO.	DOSE	TWO HOURS AFTER SUBCUTANEOUS ADMINISTRATION	AFTER THE FOLLOWING DAYS OF SUCCESSIVE ORAL ADMINISTRATION:			
			1	3	4	4½
Diphenylhydantoin-sodium	mgm./kgm.					
	25	0	—	—	—	—
	50	0	0	0	0	19
	60	0	0	0	0	30
	100	0	—	—	—	—
218	150	0	—	—	—	—
	100	—	0	0	0	22
	400	—	0	0	0	11
	300	—	0	0	0	6
	400	—	0	0	0	6
	300	—	0	0	0	17
	300	—	—	—	—	44

recognized as analogous to those muscular reactions in Jacksonian or in "grand mal" epilepsy (17). With this test, diphenylhydantoin sodium has been found by Putnam and Merritt (2), Knoefel and Lehmann (18), Bywater et al. (13), and

by myself to be effective in elevating electroshock threshold in cats; the parallelism with clinical experimentation seems to be a good criterion of the method.

An attempt has been made to obtain *quantitative* information by measuring the increase in electroshock seizure threshold. Results with phenobarbital and diphenylhydantoin-sodium are consistent.

Although convulsions arising from analeptic drugs seem to have a subcortical rather than a cortical origin, the recent works of Goodman et al. (7, 16) and Richards and Everett (6, 8), show that the antagonism against metrazol also gives a very useful index of antiepileptic activity. This technique has been used in the case of some compounds. On the other hand, failure of diphenylhydantoin-sodium to prevent chemically induced convulsions is reported in the literature. We have been able, however, to confirm in the rat the previous results of Goodman in mice (9); i.e., that after prolonged oral administration of four and one-half days in the rat, diphenylhydantoin-sodium protects against metrazol induced convulsions. These observations have been extended to phenylalkyl-

TABLE 6

Comparative activity of phenylalkylthiomethylhydantoins and diphenylhydantoin-sodium

REFERENCE NO.	PROTECTION AGAINST ELECTROSHOCK (CAT)	PROTECTION AGAINST METRAZOL CONVULSIONS (RAT)
218	25-50	50
220	12.5	12.5
221	8.3	16.6
228	10	12.5
229	16.6	16.6
230	16.6	16.6
Diphenylhydantoin-sodium . . .	100	100

thiomethylhydantoins. It seems that the saturation of the body by diphenylhydantoin-sodium or by one of its intermediary breakdown products (10, 11) is necessary to insure protection against epileptic convulsions. The saturation, however, does not occur immediately. This finding seems analogous to clinical observations of the ineffectiveness of diphenylhydantoin-sodium during the first days of administration.

Concerning the detection of a drug *specifically active against "petit mal"*, it appears from the important results of Goodman et al. (12) that this can be made only by the elevation of threshold for *EEG dysrhythmias elicitable by subconvulsant doses* of metrazol. Our results, therefore, cannot give more than an index of antiepileptic activity concerning "grand mal" or psychomotor seizures.

Concerning the animals species, the cat has been used, rather than the rabbit. Although it is more difficult to keep a healthy colony of this species, the cat is more suitable for screening tests in which the drug is given by mouth because of fewer disturbing vagaries of oral absorption after a 12-hour fast. Also, the cat shows more accurate and clear-cut criteria of various central depressant effects (analgesia, unsteadiness, and hypnosis). Compared with the rat, as far as the

metabolic behavior toward barbital is concerned, cats obviously approach more closely the response of human beings. Variation of response of anticonvulsant activity among the various animals may be the same as described for narcotic and anesthetic properties of barbital (14, 15).

(2) *Relationship between anticonvulsant activity and chemical structure.* Only provisional generalization can be made because, until having definite indication of an active group, this investigation has not been spread over the systematic list of compounds susceptible of being synthesized. Using the "Edison approach", the study has been limited to a few compounds in which existing data did not indicate exceptional toxicity.

As far as alkylthioether derivatives are concerned, a comparison, restricted, however, as above mentioned, can be made either between substituents of the same group or between different groups. In the same group it seems obvious that the increase of anticonvulsant activity is roughly proportional to the number of straight chain carbon atoms which they contain; for instance, (Ethylthiomethylene) isoamyl barbiturate is more active than 5-secondary butyl (5 ethylthiomethylene) barbiturate, which is in turn more active than (ethylthiomethylene) isopropyl barbiturate. Also, comparison of 5 ethyl-5(n-butylthioethylidene) barbiturate with 5 ethyl-5(n-butylthiopropylidene) barbiturate shows that the longer the chain, the more active the compound. 5(tertiary butylthiomethylene) 5-ethyl barbiturate, which has the highest molecular weight of the alkylthioethylbarbiturates studied up to now, shows the greatest duration of action (20 hours). These results are similar to those noted by Putnam and Merritt (3) with barbiturates; and by Bywater et al. (13) with benzoxazoles, as might be expected from Richardson's law about hypnotics. Comparison between three different groups of thioether derivatives shows that generally the alkylthiomethyl compounds appear less active than the alkylthioalkyl: 5 allyl-5(n-butylthiomethylene) barbiturate is less active than 5 allyl-5 (n Butylthioethylidene) barbiturate; Ethyl (Ethylthiomethylene) thiobarbiturate is less active than 5-(n Butylthioethylene-) 5-ethyl barbiturate.

Thiobarbiturates are generally less active and of shorter duration of action than their oxygen analogs: 5 secondary Butyl (5-Ethylthiomethylene) thiobarbiturate is less active than 5 secondary butyl (5 Ethylthiomethylene) barbiturate; 5 Isobutyl (5-methylthiomethylene) thiobarbiturate is less active than 5 Isobutyl (5-methylthiomethylene) barbiturate. This confirms observations of Merritt et al. (3, 4) using different anticonvulsants.

In the phenylalkylthiomethylhydantoin derivatives, the substitution of an alkylthiomethyl for one of the phenyls of diphenylhydantoin-sodium decreases the activity; this confirms recent results of Merritt et al. (3). The substitution by an alkylsulfonemethyl decreases the activity even more. Among the alkylthiomethylhydantoins substitution by an ethyl group brings about the most effective compound.

CONCLUSIONS

1. Fifty-five compounds of two different chemical groups: a) alkylthioether barbiturates and thiobarbiturates—(alkylthiomethyl, α alkylthioalkyl, β alkyl-

thioethyl); b) phenylalkylthiomethylhydantoin derivatives (alkylthiomethylhydantoins, alkylsulfonemethylhydantoins) have been tested following oral administration in the cat for their raising action of electroshock convulsive threshold. In addition, the phenylalkylthiomethylhydantoin derivatives have been tested after oral administration in the rat for their protection against metrazol induced convulsions.

2. Most alkylthioetherbarbiturates are effective in the cat only in doses sufficient to produce ataxia. A few compounds of higher molecular weight exhibit the greatest anticonvulsant activity independent of any depressant activity; the margin between effective dose and ataxic dose remains, however, small. With the exception of the ethyltertiary-butyl-thioethylbarbiturate, no compound has as great a duration of action as phenobarbital.

3. Among phenylalkylthiomethylhydantoins studied in the cat, the ethyl derivative is the most active; it is, however, only one-fourth as anticonvulsant as diphenylhydantoin and of shorter duration of action. The margin between effective and ataxic dose remains about the same for all these derivatives. Alkylsulfonemethylhydantoins are much less active.

4. Alkylthiomethylhydantoins have a slowly developing anticonvulsant activity following oral administration in the rat; they prevent metrazol convulsions over a period of four and one-half days repeated administration.

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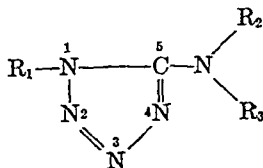
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III. SOME PHARMACOLOGIC PROPERTIES OF AMINO TETRAZOLES¹

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In the first two papers of this series (1, 2), a group of pentamethylene tetrazole derivatives and a group of 1,5-disubstituted tetrazole derivatives were studied in an effort to determine their convulsant and analeptic actions as compared with pentamethylene tetrazole and tetrazole. The third group of tetrazole derivatives, with which the present report is concerned, consists of 26 derivatives of tetrazole in which a variety of alkyl groups or the phenyl group has been introduced in position 1 and a series of mono- and disubstituted amino groups in position 5.



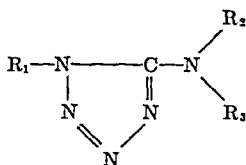
EXPERIMENTAL. The stimulatory action of these compounds upon the central nervous system was studied by the injection intraperitoneally of graded doses into albino rats of approximately equal weights. Convulsive and lethal effects were recorded. The arousal action of the drugs during pentobarbital-induced anesthesia in rats and rabbits was also studied. The criterion of arousal in rats was their ability to assume a crawling position, in rabbits, the ability to sit up and raise the head. In a few cases the ability of the compounds to increase morphine-depressed respiration in rabbits was determined by noting changes in minute volume and respiratory rate.

In several cases further studies were carried out. Compounds TT-025, TT-92, TT-023, TT-93, and TT-42 were tested for their respiratory effect on morphinized rabbits. In all cases except compound TT-92, no significant change toward normal was observed in either rate or volume of respiration when sub-convulsive

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doses were administered. Compound TT-92 increased the depth but not the rate of respiration for several minutes following the injection of the compound. The fact that these compounds are amines suggested possible vascular effects.

TABLE 1



COMPOUND NUMBER	R ₁	R ₂	R ₃	CONCENTRATION OF AQUEOUS SOLUTION OF THE HYDROCHLORIDE
TT-013	CH ₃	C ₂ H ₅	H	10%
TT-97	CH ₃	C ₆ H ₅ CH ₂	H	10%
TT-31	C ₂ H ₅	H	H	†
TT-37	C ₂ H ₅	CH ₃	H	10%
TT-95	C ₂ H ₅	C ₂ H ₅	H	10%
TT-96	C ₂ H ₅	C ₂ H ₅	CH ₃	10%
TT-049	C ₂ H ₅	C ₆ H ₅ CH ₂	H	4%
TT-047	C ₂ H ₅	C ₆ H ₅ CH ₂ CH ₂	H	†
TT-057	n-C ₇ H ₇	C ₂ H ₅	H	10%
TT-026	iso-C ₇ H ₇	C ₂ H ₅	H	10%
TT-025	n-C ₈ H ₉	C ₂ H ₅	H	10%
TT-92	iso-C ₈ H ₉	CH ₃	H	10%
TT-01	iso-C ₈ H ₉	C ₂ H ₅	H	10%
TT-023	n-C ₈ H ₁₁	C ₂ H ₅	H	10%
TT-024	iso-C ₈ H ₁₁	C ₂ H ₅	H	10%
TT-058	(C ₂ H ₅) ₂ CH	C ₂ H ₅	H	10%
TT-066	n-C ₇ H ₁₅	CH ₃	H	10%
TT-059	n-C ₇ H ₁₅	C ₂ H ₅	H	10%
TT-93	C ₆ H ₁₁ *	CH ₃	H	7.5%
TT-99	C ₆ H ₁₁ *	C ₂ H ₅	H	10%
TT-015	C ₆ H ₁₁ *	C ₂ H ₅	C ₂ H ₅	10%
TT-34	C ₆ H ₅	CH ₃	H	10%
TT-056	C ₆ H ₅	C ₂ H ₅	H	10%
TT-42	C ₆ H ₅	CH ₃	CH ₃	10%
TT-032	C ₆ H ₅ CH ₂	C ₂ H ₅	H	4%
TT-065	C ₆ H ₅ CH ₂ CH ₂	C ₂ H ₅	H	10%

* C₆H₁₁ = Cyclohexyl.

† Tested as a 5% solution of the free base.

‡ Tested as a 7% solution of the Hydrobromide.

Several of the compounds were injected intravenously into dogs with no effect on blood pressure or heart rate.

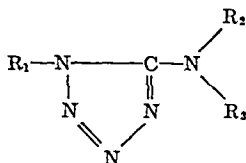
DISCUSSION. Several of the compounds in this series were found to be potent convulsant agents, but none exhibited any high degree of analeptic action, and some depressed respiration to the extent that death resulted. In fact, the most

TABLE 2
Convulsant and lethal doses

COMPOUND* NO.	ANIMAL	MINIMUM CONVULSIVE DOSE	MINIMUM LETHAL DOSE	COMMENT†
		mgm./kgm.	mgm./kgm.	
TT-013	Rat	1000	—	Maximum dose used 1500 mgm./kgm. Early depression followed by tremors
TT-97	Rat	100	150	Some resp. stimulation but no arousal with doses of 150-300 mgm./kgm. after Pb. Fatal above 300 mgm./kgm.
TT-31	Rat	No action	—	
TT-37	Rat	500	500	Maximum dose used 500 mgm./kgm.
TT-95	Rat	275	400	Maximum dose used 400 mgm./kgm. Extreme resp. depression
TT-96	Rat	120	130	Convulsant; lethal doses approximately the same
TT-049	Rat	80	150	Maximum dose used 400 mgm./kgm. After Pb. Fatal
TT-047	Rat	50	150	Maximum dose used 500 mgm./kgm. after Pb.
TT-057	Rat	250	400	Analeptic dose not determined
TT-026	Rat	400	500	Analeptic dose not determined
TT-025	Rat	60	80	Maximum dose used 500 mgm./kgm. after Pb. Latent death from doses above 200
TT-32	Rat	80	100	Momentary arousal only from 200 mgm./kgm. after Pb. Death in 3-5 min. from resp. failure
TT-01	Rat	60	120	Momentary arousal only from 200 mgm./kgm. after Pb.
TT-01	Rabbit	—	—	70 mgm./kgm. I.V. given in divided doses produced slight arousal following each injection. Resp. failure after last injection
TT-023	Rat	25	50	Maximum dose used 500 mgm./kgm. after Pb. Resp. failure 10-15 min. after 100-500 mgm./kgm.
TT-024	Rat	30	60	Maximum dose used 300 mgm./kgm. after Pb. Resp. failure above 200 mgm./kgm.
TT-053	Rat	100	150	150 mgm./kgm. fatal after Pb. No arousal
TT-066	Rat	60	60	Doses above 250 mgm./kgm. fatal after Pb. in rats. No arousal
TT-066	Rabbit	—	—	25 mgm./kgm. I.V. fatal in 4 min. No arousal. Resp. failure
TT-059	Rat	70	120	Convulsions from non-lethal doses persisted for several hours and necessitated killing the animals. Analeptic effect not determined
TT-93	Rat	180	180	Maximum dose used 800 mgm./kgm. after Pb. 200-800 mgm./kgm. fatal after Pb.
TT-99	Rat	50	100	Maximum dose used 250 mgm./kgm. after Pb.
TT-99	Rabbit	—	—	10 mgm./kgm. I.V. and 50 mgm./kgm. I.M. failed to produce arousal
TT-015	Rat	60	80	Partial arousal from 200 mgm./kgm. after Pb but death followed. 400 mgm./kgm. produced greater arousal; death in 6 min.

doses were administered. Compound TT-92 increased the depth but not the rate of respiration for several minutes following the injection of the compound. The fact that these compounds are amines suggested possible vascular effects.

TABLE 1



COMPOUND NUMBER	R ₁	R ₂	R ₃	CONCENTRATION OF AQUEOUS SOLUTION OF THE HYDROCHLORIDE
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TT-97	CH ₃	C ₆ H ₅ CH ₂	H	10%
TT-31	C ₂ H ₅	H	H	†
TT-37	C ₂ H ₅	CH ₃	H	10%
TT-95	C ₂ H ₅	C ₂ H ₅	H	10%
TT-96	C ₂ H ₅	C ₂ H ₅	CH ₃	10%
TT-049	C ₂ H ₅	C ₆ H ₅ CH ₂	H	4%
TT-047	C ₂ H ₅	C ₆ H ₅ CH ₂ CH ₂	H	†
TT-057	n-C ₂ H ₇	C ₂ H ₅	H	10%
TT-026	iso-C ₂ H ₇	C ₂ H ₅	H	10%
TT-025	n-C ₄ H ₉	C ₂ H ₅	H	10%
TT-92	iso-C ₄ H ₉	CH ₃	H	10%
TT-01	iso-C ₄ H ₉	C ₂ H ₅	H	10%
TT-023	n-C ₆ H ₁₁	C ₂ H ₅	H	10%
TT-024	iso-C ₆ H ₁₁	C ₂ H ₅	H	10%
TT-058	(C ₂ H ₅) ₂ CH	C ₂ H ₅	H	10%
TT-066	n-C ₇ H ₁₅	CH ₃	H	10%
TT-059	n-C ₇ H ₁₅	C ₂ H ₅	H	10%
TT-93	C ₆ H ₁₁ *	CH ₃	H	7.5%
TT-99	C ₆ H ₁₁ *	C ₂ H ₅	H	10%
TT-015	C ₆ H ₁₁ *	C ₂ H ₅	C ₂ H ₅	10%
TT-34	C ₆ H ₅	CH ₃	H	10%
TT-056	C ₆ H ₅	C ₂ H ₅	H	10%
TT-42	C ₆ H ₅	CH ₃	CH ₃	10%
TT-032	C ₆ H ₅ CH ₂	C ₂ H ₅	H	4%
TT-065	C ₆ H ₅ CH ₂ CH ₂	C ₂ H ₅	H	10%

* C₆H₁₁ = Cyclohexyl.

† Tested as a 5% solution of the free base.

‡ Tested as a 7% solution of the Hydrobromide.

Several of the compounds were injected intravenously into dogs with no effect on blood pressure or heart rate.

DISCUSSION. Several of the compounds in this series were found to be potent convulsant agents, but none exhibited any high degree of analeptic action, and some depressed respiration to the extent that death resulted. In fact, the most



* "R" represents the tetrazole nucleus.

** Arrows indicate direction of increased stimulatory action.

TABLE 2—*Continued*

COMPOUND* NO.	ANIMAL	MINIMUM CONVULSIVE DOSE	MINIMUM LETHAL DOSE	COMMENT†
		mgm./kgm.	mgm./kgm.	
TT-34	Rat	400	600	Produces intermittent convulsions and sedation. Analeptic effect not determined
TT-056	Rat	300	500	Analeptic effect not determined.
TT-42	Rat	100	200	Slight arousal from 500 mgm./kgm. after Pb. followed by resp. failure and death
TT-032	Rat	30	50	300 mgm./kgm. fatal without arousal after Pb.
TT-065	Rat	40	75	200 mgm./kgm. produced resp. failure but no arousal
TT-065	Rabbit	—	—	25 mgm./kgm. I.V. produced no arousal but death from resp. failure in 2 min.

* All compounds were given in aqueous solution intraperitoneally unless otherwise specified.

† Arousal was also tested following pentobarbital anesthesia but in no case were the minimum criteria for arousal obtained with the doses used.

Pb. = Pentobarbital.

potent convulsant compounds were usually the greatest respiratory depressants. This is in contrast to the actions of the active members of the pentamethylene tetrazole and disubstituted tetrazole series. It appears that the addition of an amino group in position 5 of the tetrazole nucleus leads to compounds which inhibit respiration.

Inspection of the data recorded in table 2 indicates certain relationships between changes in chemical structure and pharmacologic action which are represented schematically in the accompanying diagram.

The unsubstituted amino compound of this series, 1-ethyl-5-aminotetrazole (TT-31), showed no central nervous action, either stimulatory or depressant. The inactivity of the compound was overcome to some extent by substitution of a single ethyl (TT-95) or methyl (TT-37) group for one of the amino hydrogens, reversal of the ethyl and methyl groups (TT-013), or replacement of the ethyl group of TT-95 with a phenyl group (TT-056). In general, the convulsive activity of the compounds in this group can be markedly enhanced by increasing the size of the alkyl substituent in position 1 or by increasing the size of the alkyl substituent on the amino group in position 5. If, in the general structure as given in table 1, R_2 and R_3 remain constant, the activity of the compound can be enhanced by increasing the size of the substituent R_1 . The optimum size of R_1 appears to be an alkyl group containing 5 carbon atoms either in a straight chain (TT-023) or in a branched chain (TT-024) providing the branching does not occur too close to the point of attachment of the group to the tetrazole ring structure (TT-058). The activity of the resulting compound decreases if the alkyl group becomes either smaller or larger than a 5 carbon radical. Another method of enhancing the potency of the compound is to increase the size of the alkyl group R_2 attached to the primary amino group while R_1 remains the same

THE PHARMACOLOGIC ACTION OF CERTAIN ANALOGUES AND DERIVATIVES OF DDT¹

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The two main systemic actions of DDT are a) central nervous system effects characterized by hyperexcitability, generalized tremors, spastic or flaccid paralysis and convulsions; b) hyaline degeneration and focal necrosis of the liver (1, 2). The mechanism of action of DDT is still not too well understood. More is known about the metabolic fate of DDT, since it has been shown that the excretion product in the urine of some experimental animals is for the most part the partially dechlorinated p-p'-dichlorodiphenyl acetic acid (3, 4). The purpose of the present paper is to report experiments on the toxicity and systemic effects of a series of compounds chemically related to DDT. Such information it is believed might be helpful in providing some knowledge on the action of DDT which could not be otherwise obtained.

EXPERIMENTAL. Twelve compounds, in addition to DDT, some of which were synthesized in this laboratory and some obtained from outside sources, were studied to determine their acute toxicity in rats, and their chronic toxicity and manner of action in rabbits.

The drugs were administered orally by stomach tube in olive oil solution in all cases except where otherwise indicated. In the acute toxicity experiments white rats were used, weighing 150 to 200 grams. In the chronic experiments rabbits were used of about 2 to 2.5 Kg., kept in metabolism cages and maintained on a diet of oats and cabbage. The drugs were administered to the rabbits daily, 5 to 6 times a week, until death or until some 30 doses were given when the experiment was terminated. The survivors were then killed with chloroform, post mortem findings noted, and the tissues fixed with formaldehyde for microscopic examination.

The following compounds were studied in the course of this investigation² ($R=C_6H_5$).

- | | | |
|-----------|---|---|
| 1. DDT, | $\begin{array}{c} \text{H} \\ \\ \text{Cl}-\text{R}-\text{C}-\text{R}-\text{Cl} \\ \\ \text{CCl}_3 \end{array}$ | p,p'-dichlorodiphenyl trichloroethane (a) |
| 2. DBrDT, | $\begin{array}{c} \text{H} \\ \\ \text{Br}-\text{R}-\text{C}-\text{R}-\text{Br} \\ \\ \text{CCl}_3 \end{array}$ | p,p'-dibromodiphenyl trichloroethane (b, c) |

¹p-p'-dichlorodiphenyl trichloroethane.

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²The compounds were obtained as follows: (a) Prepared or purified in this laboratory. (b) Courtesy of Dr. V. Froelicher; Geigy Co. (c) Courtesy of Dr. H. L. Haller; Department of Agriculture. (d) Courtesy of Dr. C. J. Krister; Du Pont Chemical Co. (e) Courtesy of Dr. Alexander King of the British Commonwealth Scientific Office, Washington,

and R_3 remains hydrogen. It is to be noted in the accompanying figure that the ethylamine derivatives are generally more potent than the corresponding methylamine derivatives. The potency of a resulting compound is further enhanced if both R_2 and R_3 become alkyl groups so that a tertiary amino group occupies position 5 (TT-96, TT-42).

The substitution of the cyclohexyl group for the phenyl group of TT-056 produced a compound (TT-99) with strong convulsant activity. This activity was considerably greater in the 5-ethyl amino tetrazole than in the 5-methyl amino compound (TT-93). Still further increase of convulsant activity was obtained by substituting a benzyl group in the R_1 position (TT-032); this compound almost equalled the potency of 1-n-amyl-5-ethylaminotetrazole (TT-023). TT-032, TT-023, and TT-024 were the most convulsant compounds of the entire group.

The convulsions produced by some of these compounds, especially by TT-059, TT-058, and TT-065, were of long duration. A comparison of the most active convulsant agents of this group and the previous group of disubstituted tetrazoles (2) indicates the necessity of having the larger of the two substituted groups in the R_1 position (see compound TT-79 and TT-020 (2)).

It appears that the failure of the compounds of the present series to exhibit analeptic action must be ascribed to the presence of the amino group.

SUMMARY

The action of a group of substituted aminotetrazole derivatives on the central nervous system has been studied. The convulsant activity and the respiratory depression of the compounds appeared to be parallel. The lack of analeptic action can be ascribed to the presence of the amino group. Certain relationships between chemical structure and pharmacologic action have become apparent. The optimum structural factors for maximum central nervous stimulation appear to be the presence of a relatively large alkyl or phenylalkyl group in position 1 and a small group, such as ethyl, on the amino group in the 5 position. Some of these compounds are stimulatory in action but their lack of analeptic activity does not suggest further investigation as useful arousal agents.

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is of a very low order of toxicity. The dehydrochlorinated compound with an unsaturated carbon bond, DDD', is about $\frac{1}{10}$ as toxic, while the dichloroethane derivative, DDD, is between $\frac{1}{10}$ and $\frac{1}{20}$ as toxic as DDT. The compounds DDE and DDM, containing no aliphatic chlorine, are about $\frac{1}{10}$ as toxic as DDT while the compound DT with no aromatic chlorine is about $\frac{1}{10}$ as toxic as DDT. Of particular interest is the low toxicity of the acetic acid derivative DDA which is about $\frac{1}{10}$ as toxic as DDT on oral administration. The toxicity of this compound is considerable however when given intraperitoneally, but when compared on the

TABLE 1
Acute toxicity of DDT, analogues and derivatives in rats

COMPOUND	DOSE	ROUTE	NUMBER	MORTALITY	REMARKS
	<i>g. per kg.</i>			<i>per cent</i>	
DDT*	0.2	os	17	60	Severe tremors in 3-6 hrs., death in 18 to 48 hours.
DBrDT	0.15	os	28	50	Same
	0.3	os	11	82	
	0.2	os	12	75	
	0.15	os	12	50	
DMDT	0.1	os	12	25	Diarrhea, progressive weakness and death in 36-48 hours.
	7.0	os	13	53.8	
	5.0	os	17	0	
DDD'	2.0	os	12	75	Some hyperexcitability and mild tremors in 24 hours, death in 1 to 9 days.
	1.5	os	12	75	
	1.0	os	15	46.6	
DDD	3.0	os	16	68.7	Moderate tremors in 24 hrs., death in 1 to 12 days.
	2.5	os	10	30	
DDE	1.0	os	4	50	Death in 24 to 48 hrs. No specific symptoms.
DDM	2.0	os	10	70	Progressive muscular weakness, death in 1 to 4 days.
	1.5	os	15	73	
	1.0	os	10	50	
DDA	2.0	os	10	60	Labored respiration, muscular weakness and death in 3 to 24 hrs.
	1.5	os	10	20	
	1.0	os	10	0	
	0.2	I.p.†	13	100	All died in 2½ to 40 hrs.
	0.15	I.p.†	15	60	Death in 6 to 48 hours.
	0.10	I.p.†	10	0	
	0.2	I.p.‡	10	60	
DT	2.0	os	13	77	No specific symptoms, death in 1 to 4 days.
	1.0	os	10	20	

* From data previously published, reference 1.

† Administered as the sodium salt in aqueous solution.

‡ Administered as the acid in olive oil solution.

basis of DDT toxicity when both are given intravenously in rabbits it is about $\frac{1}{2}$ as toxic as DDT.³

³The LD₅₀ of a 0.4% solution of DDT in water containing 10% "Tween 20" (Polyalkylene derivative of sorbitan monolaurate, Atlas Powder Co.) on intravenous injection in rabbits is 30 mg. per kg. The LD₅₀ of DDA in rabbits when injected intravenously as the sodium salt is 150 mg. per kg.

3. DMDT, $\text{CH}_3\text{O}-\text{R}-\overset{\text{H}}{\underset{\text{CCl}_3}{\text{C}}}-\text{R}-\text{OCH}_3$, p,p'-dimethoxydiphenyl trichloroethane (d)
4. DDD', $\text{Cl}-\text{R}-\overset{\text{H}}{\underset{\text{CCl}_3}{\text{C}}}-\text{R}-\text{Cl}$, p,p'-dichlorodiphenyl dichloroethylene (a)
5. DDD, $\text{Cl}-\text{R}-\overset{\text{H}}{\underset{\text{CHCl}_2}{\text{C}}}-\text{R}-\text{Cl}$, p,p'-dichlorodiphenyl dichloroethane (a, b)
6. DDE, $\text{Cl}-\text{R}-\overset{\text{H}}{\underset{\text{CH}_3}{\text{C}}}-\text{R}-\text{Cl}$, p,p'-dichlorodiphenyl ethane (e)
7. DDM, $\text{Cl}-\text{R}-\overset{\text{H}}{\underset{\text{H}}{\text{C}}}-\text{R}-\text{Cl}$, p,p'-dichlorodiphenyl methane (a)
8. DDA, $\text{Cl}-\text{R}-\overset{\text{H}}{\underset{\text{COOH}}{\text{C}}}-\text{R}-\text{Cl}$, p,p'-dichlorodiphenylacetic acid (a, b)
9. DT, $\text{H}-\text{R}-\overset{\text{H}}{\underset{\text{CCl}_3}{\text{C}}}-\text{R}-\text{H}$, diphenyl trichloroethane (b)
10. DE, $\text{H}-\text{R}-\overset{\text{H}}{\underset{\text{CH}_3}{\text{C}}}-\text{R}-\text{H}$, diphenyl ethane (f)
11. DA, $\text{H}-\text{R}-\overset{\text{H}}{\underset{\text{COOH}}{\text{C}}}-\text{R}-\text{H}$, diphenyl acetic acid (f)
12. DDK, $\text{Cl}-\text{R}-\text{CO}-\text{R}-\text{Cl}$, p,p'-dichlorobenzophenone (f)
13. DDK', $\text{Cl}-\text{R}-\text{CO}-\text{R}-\text{Cl}$, o,p'-dichlorobenzophenone (f)

RESULTS. The acute toxicity of some of these compounds in rats is summarized in table 1. The LD₅₀ figures or their approximates are boldface in the table for convenience. From this it will be seen that the bromine analogue, DBrDT, is of about the same order of toxicity as DDT itself. The methoxy analogue, DMDT,

It was prepared at the Chemical Defense Experimental Station of the British Ministry of Supply. Only a small sample of this was available for this work. (f) Eastman Kodak Co.

ministered contained aromatic or aliphatic chlorine. The rate of elimination in relation to dose administered was highest for DDA, with DT, DDM, DDK, DDD and DDT in descending order. The rate of elimination of DDD' was the lowest, so low compared with DDT as to militate against the view that DDD' might be an intermediary in the metabolic degradation of DDT, despite the easy transformation of DDT to DDD' *in vitro* (5).

DISCUSSION. The two toxicologically most significant actions of DDT, the neurotoxic and hepatotoxic actions, appear to be related to the five halogens. Lauger's hypothesis attributing toxicity to the linked p-chlorobenzene rings, and lipid solubility to the trichloroethane bridge (6) does not fit in with the present data. Nor does Martin's (7) suggestion that the trichloroethane group is responsible for toxicity by splitting off HCl at vital centers. Lipid solubility alone does not account for differences in action and toxicity, since DDT is about inter-

TABLE 3

Urinary elimination of organic chlorine in rabbits receiving DDT, analogues and derivatives. Daily dose 50 mg. per kg. (except DDT which was 25 mg. per kg.; DDK' 400 mg. per kg. and DMDT 200 mg. per kg.)

Figures represent averages of 4 to 5 animals

COMPOUND	MG. ORGANIC CHLORINE PER DAY		
	2d to 3d day	10th to 12th day	20th to 25th day
DDT.....	1.4	1.8	6.7
DDD'.....	0.7	1.3	3.4
DDD.....	6.4	7.3	7.1
DDM.....	7.6	5.0	13.9
DT.....	8.8	13.1	14.1
DDA.....	10.0	14.5	17.2
DDK.....	8.5	10.1	
DDK'.....		37.5	
DMDT.....	19.2	21.4	

mediate between DDA and DDD which are about the least soluble in olive oil, and DT and DDM which are about the most soluble in the series. Nor is there a definite relationship of the toxicity of these compounds to mammals and insecticidal activity. A survey by Busvine (8) of the insecticidal properties against lice and bed bugs of some of the compounds listed here indicates that the most active in the series next to DDT is the dichloroethane derivative DDD which was from $\frac{1}{2}$ to $\frac{1}{3}$ as active, though according to our studies it is almost $\frac{1}{20}$ as toxic as DDT to mammals. The recent studies by Deonier and Jones (9) also indicate high larvicidal activity for DDD. This raises the interesting question of possible superiority of DDD over DDT in their practical application as insecticides.

SUMMARY

Studies were made of the acute and chronic toxicity of a series of compounds structurally related to DDT. The results indicate that the characteristic neu-

In this series it is noteworthy that only the two fully halogenated compounds, DDT and DBrDT, possessed a relatively high degree of toxicity and produced the characteristic tremors. Greatly reduced neurologic effects were discernible with the two compounds containing four halogen atoms, DDD and DDD', but none were seen with compounds containing three or less.

The *chronic toxicity* of the compounds in rabbits is summarized in table 2. Here again the compounds most toxic to the central nervous system and liver were DDT and the bromine analogue DBrDT. The methoxy analogue, DMDT, killed the animals after 4 to 15 doses of 200 mg. per kg. each given daily, but there were no other symptoms than diarrhea and anorexia. The dichloroethylene derivative, DDD', produced some hyperexcitability suggestive of DDT

TABLE 2

Chronic toxicity of DDT, analogues and derivatives in rabbits, 50 mg. per kg. per day

COMPOUND	NO TREAT-ED	NO OF DOSES GIVEN			NO. DIED	CNS SYMPTOMS	LIVER NECROSIS	MICROSCOPIC PATHOLOGY			
		Min	Max	Average				Fatty liver de-generation	Fatty heart	Ne-phro-sis	Hemo-sidero-sis
DDT	6	15	25	20	6	+++	6		0	0	6
DBrDT	5	4	17	11.6	5	+++	5				5
DMDT	4	4	15	7.8*	5	0	0	2	2	4	2
DDD'	5	11	28	18	5	+	0	3	2	2	3
DDD	5	20	32	27	2	0	0	0	2	0	5
DDM	5	19	32	27	2	0	0	0	0	0	4
DDA	5	7	32	24	2	0	0	0	3	4	3
DT	5	15	32	27	2	0	1	0	1	1	4
DE	5	21	34	27	2	0	0	0	1	0	4
DA	5	28	33	31	2	0	0	2	1	1	4
DDK	8	9	26	13†	8	0	0	2	1	1	3
DDK'	7	4	8	5.3‡	7	0	1	1	5	1	3

* Daily dose 200 mg. per kg.

† Daily dose varied from 50 to 250 mg. per kg. and computed on the basis of a 50 mg. average daily dose

‡ Daily dose 400 mg. per kg.

action, while the dichloroethane derivative, DDD, had no demonstrable effects in the doses given. All the other were free of neurotoxic action. The action on the liver too was either absent or slight compared with DDT or the bromine analogue. It seems that the five halogens in the molecule are essential for liver toxicity as well as for pronounced neurotoxic action, that partial dechlorination of the ethane chain greatly reduces toxicity, and that compounds with aromatic chlorine or aliphatic chlorine alone are incapable of eliciting in rabbits the type of central nervous system toxicity that is so characteristic of DDT.

Examination of the urine for organic chlorine by the method previously described (1), as shown in table 3, indicates some elimination of the compounds or their degradation products in all cases, regardless whether the compounds ad-

m.p. 163–165°, was obtained (35.3% of the calculated). The ether extract was dried with calcium chloride, the ether was distilled off, and the remaining oil, which solidified on standing, was again refluxed for one hour, using a solution of 20 g. of potassium hydroxide in 300 cc. of ethylene glycol. An additional quantity of 3.2 g. of the acetic acid compound was obtained. Repeating the foregoing process yielded another 1.3 g. The total yield was 15.75 g. = 49.5% of the calculated. The crude product (30 g.) was crystallized from hot 36% acetic acid. The solubility in the boiling acid is about 1.2%. Twenty-five grams of large, broad, colorless needles were obtained which melted at 167.5–168°.

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rotoxic and hepatotoxic actions of DDT are dependent on the five halogens in the molecule. Compounds with aromatic or aliphatic chlorine alone exhibit little toxicity and none of the DDT central nervous system actions. Compounds with partial dechlorination of the ethane bridge exhibit the neurotoxic action of DDT only to a minor degree and are relatively non-toxic. The least toxic of all the compounds studied is the methoxy analogue of DDT (DMDT).

Organically bound chlorine was found in the urine of all rabbits receiving compounds with aromatic or aliphatic chlorine in the molecule, thus indicating some excretion in the urine of the compounds or their degradation products.

APPENDIX

Notes on the preparation of some compounds related to DDT:

Compound No. 4. DDD', p,p'-dichlorodiphenyl dichloroethylene, 1,1-dichloro-2,2-bis(4-chlorophenyl)-ethylene. This compound was prepared according to Zeidler (5); refluxing for one hour instead of 10 hours was sufficient. An isomeric and labile product was obtained by carrying out the reaction at room temperature and precipitating the reaction product with water. From alcohol, the isomeric compound crystallized in large, clear prisms of m.p. 75°. After standing for several days, the crystals became turbid and showed the m.p. of the normally obtained DDD', namely 89°.

Compound No. 5, DDD, p,p'-dichlorodiphenyl dichloroethane, 1,1-dichloro-2,2-bis(4-chlorophenyl)-ethane. A satisfactory yield of this compound was obtained by condensation of dichloroacetal with chlorobenzene. Dichloroacetal was prepared according to Fritsch (10) by chlorinating alcohol. A fraction boiling at 178–188° at ordinary pressure was used for condensation. Sixty grams of chlorobenzene and 50 grams of dichloroacetal was shaken for three hours with a mixture of 450 cc. of concentrated sulfuric acid and 30 cc. of fuming H_2SO_4 containing 20–30% SO_3 . A white solid substance was formed which was separated and washed with water. The yield of the crude product was 67 g. = 78% of the calculated. From hot methanol large prisms were obtained of m.p. 109.5–110°.

Anal. Calcd. for $\text{C}_{14}\text{H}_{10}\text{Cl}_4$: C, 52.54; H, 3.15; Cl, 44.32.

Found: C, 52.47; H, 3.34; Cl, 44.36.

Compound No. 7, DDM, p,p'-dichlorodiphenylmethane. This compound was prepared according to White and Sweeney (3) with slight modification. DDT (40 g.) was refluxed with potassium hydroxide (80 g.) in a solution of ethylene glycol (1000 cc.) Upon addition of 2000 cc. of water, a colorless precipitate was obtained in theoretical yield (27 g.) Crystallized from methanol, it melted at 55–56°.

Compound No. 8, DDA, p,p'-dichlorodiphenyl acetic acid. The mode of preparation used differs from the procedure given by White and Sweeney (3) in so far as, instead of barium hydroxide, potassium hydroxide was used for hydrolysis and the reaction time considerably shortened. DDT (40 g.) was refluxed with potassium hydroxide (80 g.) in a solution of ethylene glycol (1250 cc.) for one hour. The reaction mixture was diluted with an equal volume of water, extracted with ether, filtered and acidified. 11.25 g. of the acetic acid compound,

m.p. 163–165°, was obtained (35.3% of the calculated). The ether extract was dried with calcium chloride, the ether was distilled off, and the remaining oil, which solidified on standing, was again refluxed for one hour, using a solution of 20 g. of potassium hydroxide in 300 cc. of ethylene glycol. An additional quantity of 3.2 g. of the acetic acid compound was obtained. Repeating the foregoing process yielded another 1.3 g. The total yield was 15.75 g. = 49.5% of the calculated. The crude product (30 g.) was crystallized from hot 36% acetic acid. The solubility in the boiling acid is about 1.2%. Twenty-five grams of large, broad, colorless needles were obtained which melted at 167.5–168°.

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THE EFFECTS OF SODIUM SUCCINATE AND SUCROSE DIURESIS UPON PENTOBARBITAL ANESTHESIA

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The therapy of barbiturate poisoning is an increasingly important problem in the medical field. Considerable investigation has been undertaken to determine an effective antagonist to barbiturate overdose. Soskin and Taubenhaus (1) reported that sodium succinate was an effective antidote in rats and had been used successfully in the treatment of one case of overdose of seconal and allonal in man. More recently Campbell et al. (2) reported successful therapy using succinate in cases of heavy dosage of pentothal sodium in humans. Beyer and Latven (3) have shown that sodium succinate may be of some aid in opposing the effects of barbituric acid derivatives. Lardy, Hansen and Phillips (4) found that picrotoxin was more effective than succinate in counteracting pentobarbital.

The barbiturates have antidiuretic effects if the neurohypophysis is functioning (de Bodo and Prescott, 5). Johnson et al. (6) and Gower et al. (7) found some diuretic agents effective in treatment of barbital poisoning. Corson et al. (8) reported that increased excretion of barbital and pentobarbital accompanied diuresis during succinate or fumarate treatment. In many cases, however, there was no specific beneficial effect. If the diuretic action of succinate is primary, the variation in the results obtained may be due in part to the mode of injection, which would affect the rate of onset and degree of diuresis.

In the present investigation intravenous and intraperitoneal injections of succinate and intravenous injections of sucrose are compared with respect to their effects upon duration of pentobarbital anesthesia and upon urinary excretion in the dog.

PROCEDURE. Anesthesia was produced by injecting into the saphenous vein sufficient freshly prepared pentobarbital sodium (60 mgm./cc.) to give 30 mgm./kgm. of body weight. Sodium succinate was prepared immediately before using, and a warmed 50% solution was injected either intravenously or intraperitoneally so that the animal received 1 gram/kgm. of body weight. The sucrose was prepared in a similar manner except where the dose was 2 gram/kgm. of body weight, in which case the solution injected was 1 gram/cc. so that the quantity of fluid given remained the same. The sleeping time of animals receiving an injection of succinate or sucrose was compared with the period of anesthesia of all animals given pentobarbital only. Many of the dogs were used as controls at one time and as experimental animals at another with a period of two or more weeks intervening. In a few cases the same dog was used for both succinate and sucrose experiments. Compari-

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sons could, therefore, be made between experimental and control dogs and between the results obtained at different times with the same animal.

In 27 dogs the ureters were cannulated following the barbiturate injection. In no instance did this take more than 20 minutes. Upon completing the cannulations, succinate was administered intravenously to 5 dogs and intraperitoneally to 6 dogs. Sucrose was injected intravenously into 11 dogs, 6 with 1 gram/kgm. and 5 with 2 gram/kgm. The diuretic effect in each case was determined quantitatively and comparison was made with the urinary excretion of 5 controls. In these acute experiments, after an initial 3 hour period, the dog was given additional pentobarbital at the rate of 10 mgm./kgm. of body weight every 30 minutes until death occurred. The total amount of barbiturate necessary to cause death was compared in experimental and control dogs.

RESULTS. *The effects of succinate and sucrose upon the sleeping time of pentobarbital.* The duration of anesthesia, which was calculated from the time of injection of pentobarbital until the animal could stand without aid, ranged from 184 to 497 minutes with an average of 333 minutes for all controls. Seventeen (Nos. 10-26) of the 38 animals used in this portion of the work were obtained at the University of Arkansas, Little Rock, and the remainder were obtained from areas around the University of Missouri, Columbia. Although the conditions of the experiments were as nearly identical as possible, it was noted that the average and the range of sleeping time was considerably different for the animals of these two places. Therefore, the average of the controls obtained in Arkansas, which was 275 minutes, was used in comparisons with the experimental animals from that area, while the average of the Missouri dogs, which was 384 minutes, was compared with the data from experimental animals obtained there. The tabulations of the data on sleeping time do not include the averages of each group of experiments.

Dogs which received an intravenous injection of sodium succinate in addition to pentobarbital slept from 110 to 275 minutes with an average of 197 minutes (table 1). When comparison is made with the sleeping time of the same animal on a different day, the duration of pentobarbital anesthesia concomitant with succinate ranges from 62% to 98% with an average of 80% of the control. When compared with the normal sleeping time of all animals, the range is 40% to 100% with an average of 64% of the control sleeping time. Dogs which received an intraperitoneal injection of succinate in addition to pentobarbital by vein slept from 186 to 525 minutes with an average of 314 minutes. Compared with the control sleeping time of the same dogs, a decrease of only 6% was noted. Compared with the average control time of all dogs, the decrease was 18%.

Eleven dogs were injected with sucrose 1 gram/kgm. and 5 dogs with 2 gram/kgm. intravenously in addition to pentobarbital. The sleeping time of those animals receiving the smaller quantity of sucrose ranged from 200 to 510 minutes with an average of 336 minutes (table 2). A comparison of these results with the control time of the same animals indicates that this smaller amount of sucrose reduces the sleeping time about 7%. Compared with the average normal sleeping time of all dogs of the same area, the sleeping time was about 10% lower. The period of anesthesia of the animals given sucrose 2 gram/kgm. ranged from 258 to 398 minutes, averaging 330 minutes. These data compared with the sleep-

TABLE 1

The effect of sodium succinate upon the duration of pentobarbital anesthesia

Sodium succinate 1 gram/kgm. was given immediately following intravenous injection of pentobarbital 30 mgm./kgm. The sleeping time is reported in minutes.

PENTOBARBITAL ONLY		PENTOBARBITAL AND SODIUM SUCCINATE							
Dog no.	Sleeping time	Dog no.	Control time	After succinate given I.V.	Per cent of control	Dog no.	Control time	After succinate given I.P.	Per cent of control
10	273	11	310	260	84	36	—	330	—
13	293	12	240	188	78	37	461	525	113
14	211	16	375	263	70	39	282	305	108
25	351	17	282	275	98	41	238	207	87
38	431	18	202	125	62	42	213	190	89
40	497	19	281	230	82	43	—	186	—
59	342	20	229	160	70	50	445	381	86
61	495	21	288	250	87	53	442	377	85
62	454	22	184	167	91	54	489	350	72
		23	—	110	—	63	—	290	—
		26	—	140	—				
Average.....				197	80			314	94

TABLE 2

The effect of sucrose upon the duration of pentobarbital anesthesia

Sucrose was given intravenously immediately following intravenous injection of pentobarbital 30 mgm./kgm. The sleeping time is reported in minutes.

SUCROSE 1 GRAM/KGM.				SUCROSE 2 GRAM/KGM.			
Dog no.	Control time	After sucrose	Per cent of control	Dog no.	Control time	After sucrose	Per cent of control
15	356	255	76	50	445	258	58
23	—	200	—	52	428	302	71
27	—	402	—	53	442	368	83
44	322	350	109	54	489	398	81
45	—	288	—	55	294	375	111
47	310	368	119				
50	445	282	63				
53	442	510	115				
54	489	440	90				
55	294	223	76				
63	—	379	—				
Average.....		336	93			330	81

ing period for the same dogs or for the controls gives an average of about 80% of the normal time, or an effect comparable to that obtained with succinate given intravenously.

The initial effect of the injection of the sodium succinate at the rate of 30 cc./minute was a temporary increase in pulmonary ventilation. Kymograph

records obtained from acute experiments with 3 dogs indicated a temporary decrease in the blood pressure immediately after the injection. Slowly injected succinate was followed by no change in blood pressure and little effect upon respiration was noted. Sucrose injections of 50% solutions were not followed by any visible changes in the respiratory or vascular systems. Following the stronger solution of sucrose, sporadic changes in respiration occurred and these animals appeared sluggish for several days following the injection. The intraperitoneal injections were not followed by any visible changes in the animals.

The effects of succinate and sucrose upon diuresis. In all cases following intravenous succinate and in the majority of the animals receiving sucrose, enuresis occurred during the later part of the sleeping period. This was noted in a few of the animals given succinate via the abdominal cavity. In the 27 animals in which the ureters were cannulated after the barbiturate injection and before the succinate or sucrose injection, the results, as shown in table 3, indicate that sodium succinate intravenously in the dosage used exerted a considerable diuretic effect. The average amount of urine excreted by the control dogs was 33 cc. as compared to 170 cc. for the animals given succinate. Comparison of the amounts of urine per kilogram of body weight or per kilogram per minute of anesthesia also emphasizes the marked diuretic action of succinate when given intravenously. Approximately 50% of the urine excreted was obtained during the first 2 hours of anesthesia. Sucrose given 1 gram/kgm. of body weight was followed by moderate diuresis, most of which also occurred during the first 2 hours of anesthesia. Sucrose given 2 gram/kgm. was followed by marked diuresis the first 2 hours and a total urinary excretion comparable to that with intravenous succinate. The intraperitoneal injection of sodium succinate, however, was followed by the excretion of a smaller quantity of urine chiefly after the first 2 hours of anesthesia as in the controls. The data (table 3) shows little evidence to suggest that the diuretic action of succinate or sucrose affected the total amount of pentobarbital necessary to cause death. Dogs 19, 21 and 26 should be compared with control dog 11 as these animals were used in Arkansas. The remainder were obtained in Missouri.

DISCUSSION. The factors responsible for individual variation of pentobarbital anesthesia and especially between groups of dogs from different localities are not known. In the same adult dog under regulated conditions, the normal sleeping time does not differ significantly from time to time. Until the factors involved in individual variation are known and controlled, comparisons should be based wherever possible upon data from the same dog under different conditions.

The effect of sodium succinate upon pentobarbital anesthesia in rats has been variable. An analysis of the work of Soskin (1), Beyer (3), Pinschmidt (9), and their associates indicates that succinate is most effective in decreasing sleeping time when given intravenously and less effective when given intramuscularly or intraperitoneally. In some instances large intraperitoneal injections of succinate are detrimental, increasing the duration of anesthesia (Lardy et al., 4) or decreasing the lethal dose (De Boer, unpublished). Corson et al. (8) found that, although the diuretic effect of succinate was accompanied by increased ex-

TABLE 3

The effect of sodium succinate and sucrose upon the excretion of urine

An initial intravenous injection of pentobarbital 30 mgm./kgm. was followed within 20 minutes by a single injection of succinate or sucrose. After 3 hours additional pentobarbital 10 mgm./kgm. was given every 30 minutes until death.

EXPERIMENTAL CONDITION	DOG NO.	URINE EXCRETED DURING ANESTHESIA						LETHAL AMOUNT OF PENTOBAR- BITAL, MGM./KGM.
		0-2 hr.	2-4 hr.	4-death	Total, cc.	No. cc./kgm.	No. cc./ kgm./min.	
Controls	11	11	13	7	31	4.43	.013	100
	27	—	—	—	43	5.97	.021	70
	28	—	—	—	52	6.50	.024	70
	29	2	7	1	10	1.54	.006	60
	30	12	16	2	30	3.13	.012	70
Average.....		8.3	12	3.3	33	4.31	.015	74
Sodium succinate I.V., 1 gram/kgm.	19	78	46	66	190	28.79	.092	90
	21	35	27	26	88	8.98	.022	120
	26	53	5	9	67	11.96	.039	100
	31	154	88	1	243	27.30	.094	70
	32	182	73	9	264	29.15	.100	70
Average.....		100	48	22	170	21.24	.070	90
Sodium succinate I.P., 1 gram/kgm.	44	10	15	7	32	6.67	.025	80
	47	16	17	7	40	5.71	.020	80
	51	36	44	19	99	14.56	.050	80
	56	3	9	6	18	4.28	.014	80
	57	7	16	9	32	4.51	.016	80
	58	29	15	2	46	7.19	.028	70
Average.....		17	18	8	45	7.14	.025	78
Sucrose I.V., 1 gram/kgm.	33	58	37	6	101	9.62	.037	70
	34	30	18	2	50	5.49	.023	70
	35	37	23	4	64	9.70	.038	70
	46	62	26	27	115	15.54	.042	100
	48	31	26	8	65	10.83	.040	70
	49	22	13	12	47	8.39	.023	100
Average.....		40	24	10	74	9.93	.034	80
Sucrose I.V., 2 gram/kgm.	60	94	33	11	138	15.68	.047	90
	64	98	31	8	137	15.75	.055	70
	66	55	28	9	92	13.73	.050	70
	67	98	31	19	148	17.01	.050	90
	68	85	24	14	123	13.10	.043	80
Average.....		86	29	12	128	15.05	.049	80

cretion of barbital and pentobarbital, there was no association with early recovery in rats, rabbits, cats, or dogs.

The present results emphasize the importance of method of administration of succinate. Intraperitoneal injection of sodium succinate 1 gram/kgm. produced little effect upon urine excretion or duration of pentobarbital anesthesia. Intravenous injection produced marked diuresis and decreased the sleeping time about 20%. These effects appear to be associated since intravenous injection of sucrose in approximately the same molar concentration (2 gram/kgm.) greatly increased urinary excretion above the normal and decreased sleeping time to the same extent as succinate. There is no strict correlation, however, between the amount of urine excreted and the decrease in sleeping time. Occasionally intraperitoneal injection of succinate produced essentially the same effect as intravenous administration (table 1, dog no. 54; table 3, dog no. 51). In these cases absorption was apparently rapid enough to produce an effect during the period of anesthesia.

In comparing the results of the present investigation with those of Corson et al., it must be pointed out that in most of their experiments the amounts of barbiturate given was sufficient to cause death in some of the animals. In the acute experiments reported here neither succinate nor sucrose increased the resistance of the animal to pentobarbital. It would seem that succinate is not beneficial when lethal or near-lethal doses of barbiturate are given. It should be pointed out also that although the quantity of succinate given was substantially the same in both series of experiments, Corson and his associates used dilute solutions administered over a long period of time. It appears that a single injection of a concentrated solution is more effective in decreasing the period of pentobarbital anesthesia.

Sucrose as a diuretic is known to dehydrate the body. If anhydremia occurred with sucrose or succinate it did not appear to affect the sleeping time adversely. Wilmer (10) observed that intravenous injections of 20% sucrose solution to give 3-4 gram/kgm. in rabbits produced hydropic degeneration of the kidney in 24 hours. The quantity used in these experiments was probably insufficient to cause kidney damage, but the concentrated solutions of sucrose might not be desirable in reducing the duration of anesthesia of longer acting, kidney-eliminated barbiturates. The effect of succinate on the kidney is not known.

Because of its lower molecular weight succinate was easier to administer than sucrose in amounts necessary to produce the same effects. The dogs receiving sucrose appeared sluggish for a few days following the experiment. The dogs given succinate showed no after effects.

SUMMARY

Intravenous sodium succinate was more effective in counteracting pentobarbital anesthesia than intraperitoneal injection of the same quantity of succinate.

Intravenous sucrose in large quantities was followed by a decrease in sleeping time comparable to that with intravenous succinate.

Intravenous succinate or sucrose resulted in immediate diuresis. Intraperi-

toneal succinate was followed by a delayed excretion of urine similar to the anti-diuretic effects of the control animals.

The length of pentobarbital anesthesia appears to be correlated in general with the degree of diuresis produced by the various drugs used and the method of injection.

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THE EFFECT OF α -TOCOPHEROL PHOSPHATE, DIGITOXIN AND CERTAIN COMPOUNDS RELATED TO THE LATTER ON CARDIAC MUSCLE METABOLISM IN VITRO

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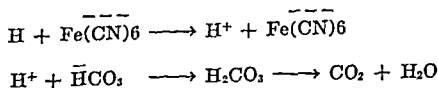
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The hypothesis of cardiac muscle anoxia in congestive heart failure, on the basis of poor diffusion of oxygen into hypertrophied muscle fibers, seems well accepted (1). It is known that anoxia of heart muscle (at least sudden anemic anoxia) produces marked destruction of coenzyme I (2), which is necessary for the proper metabolism of lactate, the preferred substrate of heart muscle (3). Thus it would seem that anoxia of heart muscle should produce marked disturbances in lactate metabolism and perhaps that the function of cardiac glycosides could be concerned with the function of lactic dehydrogenase or a part of the system in which the enzyme functions.

We have seen that α -tocopherol phosphate (α -TPh) produces marked inhibition of succinoxidase (4, 5) and, in unpublished work, the same effect has been noted in the lactic dehydrogenase system. Since α -TPh is apparently a regulator of muscle metabolism and, as will be seen, its presence or absence markedly influences the *in vitro* effects of cardiac glycosides, and since E-deficiency may produce cardiac lesions (6), one of the functions of vitamin E will be considered in this paper.

METHODS. Male guinea pigs were used as experimental animals. When desired, avitaminosis E was produced by maintaining them on diet 13 of Goettsch and Pappenheimer (7) or diet II, of Holmes and Pigott (8) for three weeks. The animals were killed by decapitation, the hearts removed, and 10% homogenates in distilled water were prepared.

All determinations of lactic dehydrogenase activity were carried out in Summerson constant volume differential manometers. The usual technique, using methylene blue as carrier and cyanide as keto-acid fixative in an atmosphere of air was used for aerobic determinations. The system was investigated anaerobically by the ferricyanide technique of Quastel and Wheatley (9), using bicarbonate buffer and an atmosphere of 95% N₂-5% CO₂. The principle of this technique depends upon the reduction of ferricyanide in bicarbonate media with the production of CO₂ thereby, according to the following equations:



All of the components of the systems except lactate were placed in both vessels of the manometers, lactate being added to only the right hand vessel. Thus basal tissue respiration not due to lactate oxidation is not recorded in the differential reading.

¹ The authors wish to express their appreciation for the helpful criticisms and suggestions of Dr. Karl H. Beyer.

All solutions except buffer were freshly made before each experiment. Digitoxin (Ladox) was prepared as a 0.01% solution in 25% alcohol, and diluted further with distilled water. Coenzyme I was prepared by the method of Williamson and Green (10), and assayed by the method of Haas (15). Various preparations of from 29 to 60% purity were used. Sodium dl- α -tocopherol phosphate was prepared in the Dept. of Organic Chemistry of this Division.² Merck digitoxin was used. Crystalline estrone and testosterone were supplied by Dr. E. G. Shipley of this Department. The ouabain employed was U.S.P. reference standard.

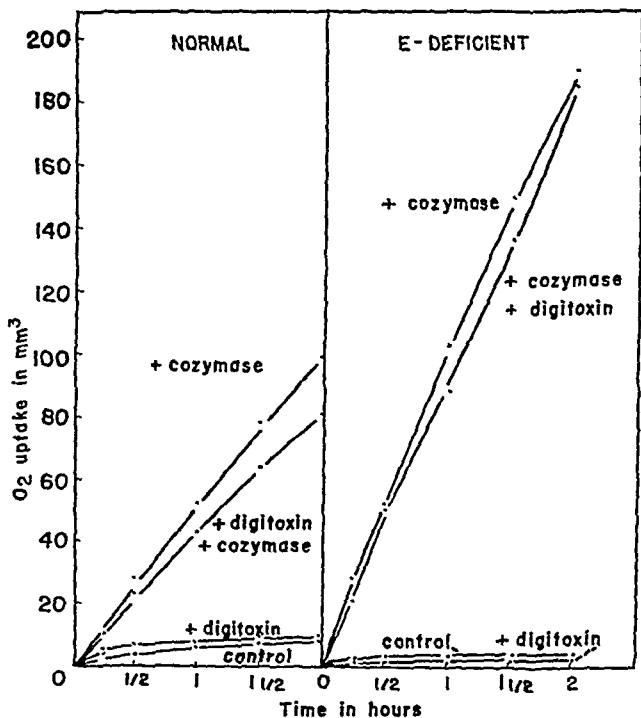


FIG. 1. Each vessel contained the following: 10% heart muscle homogenate, 0.5 cc.; NaCN, 2M, 0.1 cc.; methylene blue 0.5%, 0.1 cc.; dimethyl glycine buffer M/2, pH 8.5, 1.0 cc.; water to make 3.0 cc. When indicated the following were added: digitoxin, 0.01%; cozymase, 1.0 mg. in 0.5 cc.; sodium lactate, M/2 pH 8.5, was added to the side bulb of the right vessel in the amount 0.2 cc. and tipped in after equilibration. Center wells contained 0.1 cc. of KOH, 0.002M, and 0.1 cc. NaCN, 2M. atm.-air. $T = 37^{\circ}$.

RESULTS AND DISCUSSION. Work was begun on this problem in the early spring months when the experimental animals were fed a diet of BB rabbit pellets with very little green food. At that time we obtained results that could not be duplicated later on when the guinea pigs were fed an abundance of green food. Consequently some groups of animals were placed on the E-low diets mentioned above, with daily gavage of a solution containing 5 mg. of ascorbic acid. These

² We are indebted to Dr. E. M. Schultz for this preparation.

animals behaved as did those not fed green food, whereas animals fed pellets plus lettuce daily showed the reaction seen in the later Spring and Summer. Consequently the results will be divided into those obtained from normal and those obtained from E-deficient pigs.

The effect of digitoxin added to heart muscle homogenates *in vitro* was investigated first. As will be seen in figure 1, its addition produced no significant alteration in the aerobic system when either normal or E-deficient tissues were

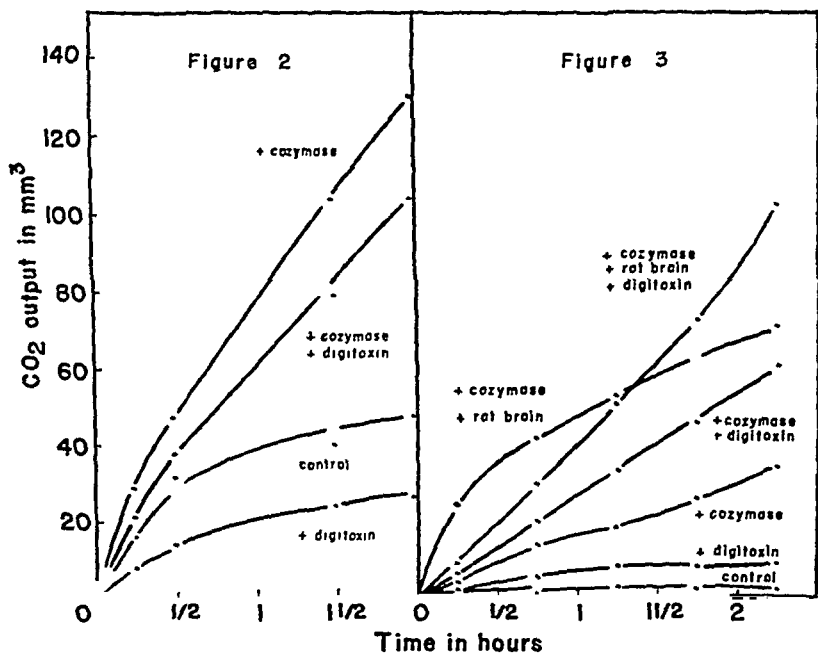


FIG. 2. Each vessel contained the following: 10% heart muscle homogenate, 0.5 cc.; NaCN, 2M, 0.1 cc.; NaHCO_3 0.025M, 1.0 cc.; cozymase, lactate, and digitoxin as in fig. 1. Side bulbs contained 0.2 cc. of 8.3% $\text{K}_3\text{Fe}(\text{CN})_6$ and were tipped after equilibration. Center wells contained yellow phosphorus. Atm. 95% N_2 -5% CO_2 . $T = 37^\circ$.

FIG. 3. Vessel contents as in fig. 2. Rat brain suspension was made as a 10% homogenate in distilled water.

used. When, however, the drug was added to a lactic enzyme system functioning anaerobically we noticed a marked stimulation of the system when coenzyme I was added, with no significant effect in the absence of added cozymase. This effect was seen only when the animals were deficient in vitamin E. Hearts from normal guinea pigs showed no effect or an inhibition when digitoxin was added to the system. Figure 2 shows the results obtained with a normal, and figure 3, with an E-deficient animal. It should be noted that digitoxin seemed to have a stimulatory effect in the E-deficient anaerobic lactic system that was roughly

proportional to the amount of coenzyme I supplied. Thus it would seem to have been protecting the coenzyme I against breakdown, rather than acting directly on the dehydrogenase. The breakdown of coenzyme I anaerobically has been described by several workers and nicotinamide has been used as an inhibitor of coenzyme I nucleotidase, the enzyme responsible for the breakdown (11). We have added nicotinamide to the E-deficient anerobic lactic dehydrogenase system, with an effect similar to that of digitoxin (fig. 4). Mann and Quastel (11a) have described rat brain as a particularly potent source of coenzyme

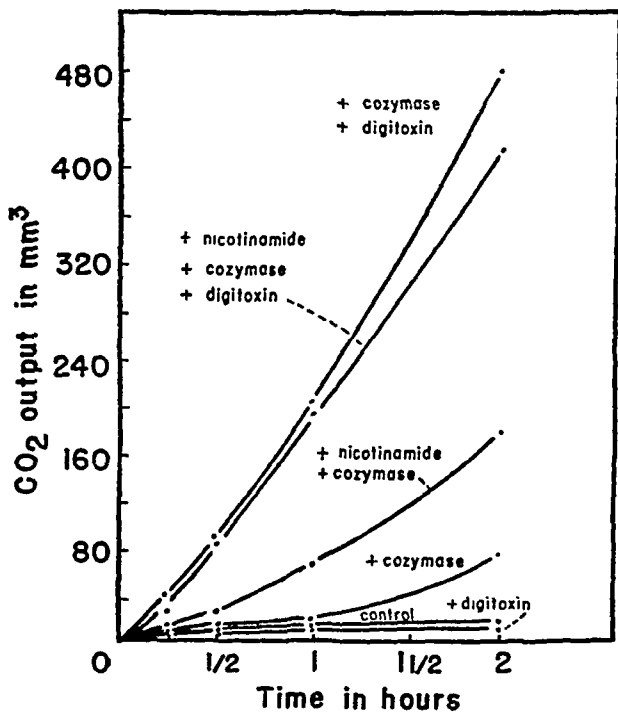


FIG. 4. Vessel contents as in fig. 2. Nicotinamide added as 0.1 cc. of 0.3% solution.

I nucleotidase. In an effort to obtain some information as to whether or not the enzyme in heart muscle is the same as that of brain, we added rat brain homogenate to the anaerobic lactic system. Here, also, digitoxin was able to increase the rate of lactate oxidation, although the rates of those vessels containing rat brain were higher due to the addition of a certain amount of cozymase in the brain suspension (fig. 3).

α -Tocopherol phosphate was tried in systems similar to those described above. No effect was seen in aerobic normal, aerobic E-deficient, or anaerobic normal

systems. In anaerobic E-deficient systems a slight stimulation usually was seen in the absence of added cozymase, with an inhibition when cozymase was added (fig. 5). It occurred to us that the fact that α -tocopherol phosphate inhibits the complete lactic dehydrogenase system need not preclude its inhibiting coenzyme I nucleotidase, the latter effect being masked by the former.³ Consequently an attempt was made to assay coenzyme I in the filtrates from the boiled vessel contents after completion of the experiment, the assay being carried out by the method of Axelrod and Elvehjem (12). It was found that digitoxin,

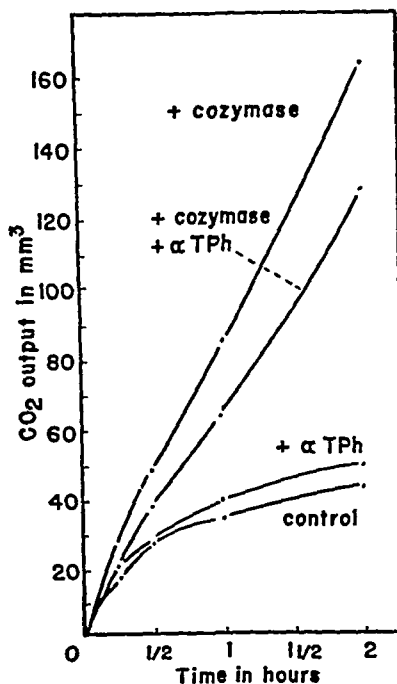


FIG. 5. Homogenate, NaCN, NaHCO₃, cozymase, lactate, ferricyanide as in figure 2. α -Tocopherol phosphate added as 0.2 cc. containing 1.0 mg. Atm. 95% N₂-5% CO₂.

α -tocopherol phosphate, and ferricyanide all inhibited the yeast apozymase, making results unpredictable. It was not found feasible to use the aerobic lactic system as a test system since ferricyanide reacts with methylene blue, producing erratic results.

When boiled tissue extracts were added to an anaerobic lactic enzyme system,

³ Later experiments have shown that if guinea pigs are *very severely* depleted of vitamin E, the inhibitory effect of α -TPh on coenzyme I nucleotidase can be shown in the lactic enzyme system as an increase in CO₂ output when α -TPh is added to vessels containing coenzyme I.

we could not be sure that digitoxin or α -TPh carried over from the previous experiment was not stimulating or inhibiting the system, so this trial was abandoned.

Consequently we decided to use the succinoxidase system as an indication of the integrity of coenzyme I. It is well known (13) that coenzyme I inhibits succinic dehydrogenase by catalyzing the formation of oxaloacetate from malate. Thus the integrity of coenzyme I could theoretically be demonstrated by noting the degree of inhibition of succinic dehydrogenase. The determination of succinic dehydrogenase activity in this case would necessarily need to be carried out anaerobically since as we have seen above, the coenzyme I nucleotidase of guinea pig heart is not active under aerobic conditions. Consequently the ferricyanide technique was applied to the measurement of succinic dehydrogenase activity of normal and E-deficient guinea pig heart.

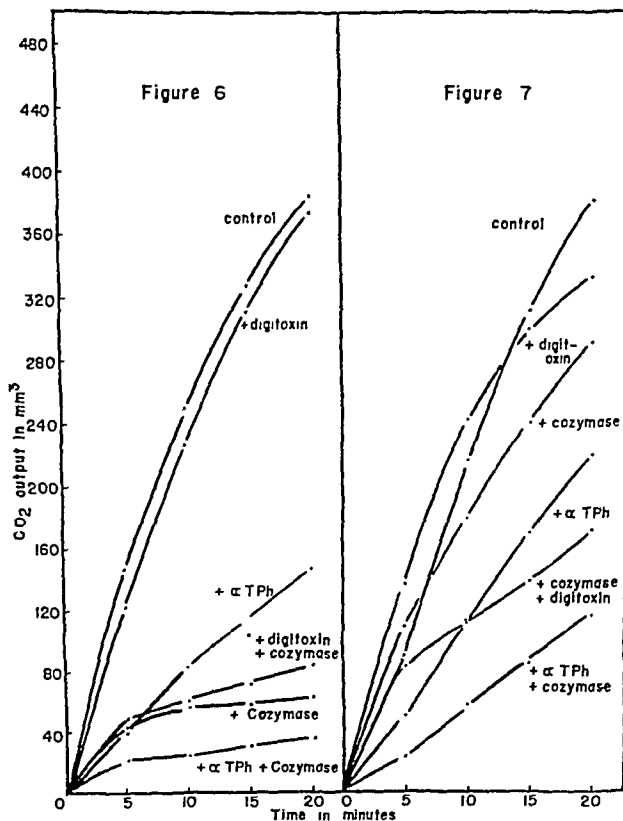
Figure 6 shows that the addition of digitoxin or of α -TPh to the normal anaerobic succinic dehydrogenase system does not influence the inhibition produced by cozymase addition. When E-deficient tissues are used, however (fig. 7) coenzyme I alone produced little inhibition, showing it to be broken down probably by the nucleotidase active in this case. The addition of α -TPh or digitoxin markedly increases the inhibition produced by cozymase, showing the nucleotidase to be inhibited by these substances. The addition of α -TPh to these systems results in inhibition of the succinic enzyme, which is not surprising, since we have already reported (5) that α -TPh diffuses out of liver homogenates and that the addition of α -TPh produces an inhibition of succinoxidase under aerobic conditions. This effect is apparently separate from the effect on coenzyme I nucleotidase, but apparently more α -TPh is required to inhibit the succinic or lactic enzymes, than is necessary for the inhibition of coenzyme I nucleotidase.

Thus it would seem that digitoxin is able to preserve coenzyme I from breakdown and that α -TPh has two effects, the inhibition of coenzyme I nucleotidase, and the direct inhibition of the succinic and lactic enzymes.

It was thought of interest to ascertain whether or not the addition of α -TPh *in vitro* could bring about the change of the picture seen in the E-deficient anaerobic lactic system to that seen in the normal system. An homogenate of E-deficient heart was divided into two portions, one portion being added to Summerson vessels containing coenzyme I and coenzyme I plus digitoxin respectively the other portion being added to similar vessels containing in addition 1.0 mg. of α -TPh. The addition of α -TPh *in vitro* removed the stimulatory effect of digitoxin (fig. 8) and substituted an inhibition.

Ouabain, testosterone, estrone, digitonin, and cholesterol, added in the same amounts as was digitoxin, were tested as inhibitors of the coenzyme I nucleotidase of heart muscle in a few experiments. All of these compounds were found active, the sex hormones probably to a lesser degree than the others. Estrone apparently has very slight activity as an inhibitor of the nucleotidase, but is quite active as an inhibitor of the lactic dehydrogenase system. In view of the recent reports of the efficacy of sex hormones (16) in the treatment of vascular

diseases, our findings are not surprising. The action of digitonin is more difficult to explain, since, although it has the cyclopentanoperhydrophenanthrene nucleus of digitoxin it is generally considered to be inactive as a cardiac glycoside. One should consider however, that the usual determination of activity of a cardiac



FIGS. 6 AND 7. Each vessel contained the following: 10% heart muscle homogenate, 0.5 cc.; NaHCO_3 0.025M, 1.0 cc.; when indicated, cozymase 1.0 mg. in 0.5 cc. digitoxin 0.01%, α -TPh 1.0 mg. in 0.2 cc. Sodium succinate M/2, 0.2 cc. in the right hand vessel of each manometer. Water was added to make 3.0 cc. total. Side bulbs contained 0.2 cc. of 8.3% $\text{K}_2\text{Fe}(\text{CN})_6$ and were tipped after equilibration. Center wells contained yellow phosphorus. Atm. 95% N_2 -5% CO_2 . $T = 37^\circ$.

drug involves the use of the intact heart, whereas in our experiments cell wall barriers have been eliminated.

Cholesterol was tried since the administration of this substance is known to produce cardiac hypertrophy in animals as well as an increased level of cholesterol in the heart itself (17) and since E-deficiency is also known to produce a high level of muscle cholesterol (18). The fact that it is of a high level of activity in

the system described here lends support to the concept of an interrelationship between cholesterol and vitamin E in muscle metabolism (20). It would seem reasonable to suppose that, since E-deficient muscle has a high rate of oxygen uptake and since cholesterol inhibits coenzyme I nucleotidase, the preservation of coenzyme I might be related to the increase in metabolic activity.

Further work on the compounds mentioned above other than digitoxin and α -TPh is in progress.

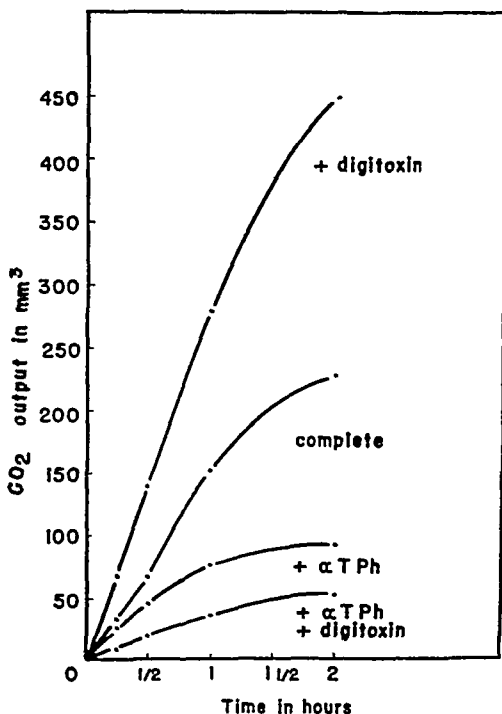


FIG. 8. Vessel contents as in figure 5 except for cozymase, of which 5 mg. were added to each vessel.

On the basis of these findings we should like to suggest an hypothesis as to a possible biochemical lesion in congestive heart failure. One might propose that chronic anoxia may produce a defect in the intracellular linkages of α -Tocopherol (of which little is known) so that a relative E-deficiency may be produced in heart muscle in the same manner in which coenzyme deficiencies are produced in tissues in shock (14). Both E-deficient muscle and cardiac muscle in congestive failure are known to show low creatine levels, which fact might suggest avitaminosis E in both cases (19). This relative deficiency might then allow functioning of coenzyme I nucleotidase in a system already anoxic, resulting in the breakdown of coenzyme I which is known to occur in

heart muscle (2). The coenzyme I breakdown would almost certainly result in serious damage to heart metabolism. In view of the findings described above, digitoxin could be expected to preserve coenzyme by the inhibition of coenzyme nucleotidase, thus allowing a more normal metabolism. α -Tocopherol could be expected to accomplish the same end. Neither compound could be expected to influence the normal heart by this mechanism.

CONCLUSIONS

1. The addition of digitoxin *in vitro* to an anaerobic E-deficient lactic dehydrogenase system of guinea pig heart results in marked stimulation of the system. This does not occur in an anaerobic lactic dehydrogenase system of normal heart muscle or in aerobic lactic enzyme systems, normal or E-deficient.

2. This effect probably is due to the inhibition of coenzyme I nucleotidase by digitoxin.

3. α -Tocopherol phosphate also protects coenzyme I against breakdown, although the rate of activity of the non-E-deficient lactic dehydrogenase system is slowed by its addition.

4. Ouabain, digitonin, cholesterol, estrone and testosterone have effects similar to those of digitoxin, to a lesser degree in the case hormones.

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RESPONSE OF BONE MARROW EXPLANTS TO LIVER EXTRACT PREPARATIONS

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Prior to 1926 no satisfactory therapeutic measure was available for the treatment of pernicious anemia. Following the work of Minot and Murphy(1), considerable experimental work has been done in an effort to isolate and determine the chemical nature of the active principle or principles. Progress in this field has been impeded seriously by the lack of an adequate method of assay for the active substance.

Several attempts have been made to develop such an assay. Jacobson (2) suggested that the reticulocyte response of normal guinea pigs to liver extract could be used as an indication of the potency of the extract. This finding has not been confirmed by other workers (3, 4). Various attempts (5, 6, 7) were made by feeding or injection of a toxic compound, to produce an anemia which would respond to liver therapy. Recently, Jacobson and Williams (8, 9) have reported that the reticulocyte response of splenectomized rabbits to liver extracts can be used as a measure of the antipernicious anemia activity of the extracts. In the first paper (8) they stated that the maturation of megaloblasts in cultures of guinea pig bone marrow might also be used as a means of detecting the presence of the antipernicious anemia principle in extracts.

Overbeek et al (10, 11) described a method of assay using a culture of explants of guinea pig bone marrow in heparinized rabbit plasma. Liver extract was said to stimulate the migration of cells (chiefly leucocytes) from the explants, not as a general effect, but to give a maximum area of migration at a concentration of liver extract, usually of the order of 0.010 U.S.P. units per cc. This effect was said to be duplicated for the same extract at a concentration approximating 0.00010 U.S.P. units per cc. Pace and Fisher (12), using a modification of this method, reported that the activity of different extracts could be represented as a linear function of the dilution at which peak migration occurred and presented a graph relating this dilution to the activity of the extract in U.S.P. units. Young and Bett (13) were unsuccessful in attempts to duplicate Pace and Fisher's work either quantitatively or qualitatively. They concluded that, under the conditions used, no difference sufficiently marked to be used as the basis of an assay method seemed to exist between the response of the bone marrow explants to active and inactive extracts or to extracts of differing clinical activity.

Personal communication from Dr. E. D. Campbell of the Eli Lilly Co. indicated a possible relationship between leucocyte migration and liver extract concentration at higher concentrations of liver extract than Fisher had suggested. The following is an attempt to characterize the response observed at the higher level, when liver extract is added to cultures of bone marrow.

In a previous paper (13), sodium oxalate had been used as an anti-coagulant.

Neither this nor citrated rabbit plasma was entirely satisfactory. Heparin as an anti-coagulant in rabbit plasma showed considerable variation from rabbit to rabbit. Difficulties were also encountered at times in obtaining sufficient blood from the animals for the experiments. For these reasons the agar-serum medium used in the culture of *Rickettsiae* was tried (14). After a few preliminary experiments it was found necessary to modify some of the details of the method to overcome some of the technical difficulties encountered in applying it to the bone marrow test.

METHOD. In the following procedures aseptic technique was observed only where noted. A modified Tyrode's solution was used as a diluent (8.00 grams NaCl, 0.20 grams KCl, 0.20 grams CaCl_2 , 0.10 grams $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 grams $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and 1.00 grams glucose, per liter).

A three per cent agar solution was made in doubly distilled water. This was filled in small bottles (30 cc. per bottle) and autoclaved at fifteen pounds pressure for fifteen minutes. A double strength modified Tyrode's solution was prepared with doubly distilled water. 300 cc. of this was mixed with 200 cc. of sheep serum. The whole was then passed through a Seitz filter and was filled into small bottles (15-20 cc. per bottle) observing sterile precautions. This solution provided a reasonable amount of a uniform medium which could be used in several tests. Sheep serum was used because it was more readily available.

To carry out the test the following dilutions of liver extract were prepared with modified Tyrode's solution: 0.001, 0.004, 0.007, 0.010 U.S.P. units per cc. In some cases five dilutions were prepared rather than four. 1 cc. of the liver dilutions was placed in each of eight culture dishes (5 cm. diameter, 1 cm. in depth). These dishes were then placed in the incubator at 37°C.

The femurs were removed from two or more guinea pigs for each test. The bone was scraped clean and the marrow removed. This was placed on a board in a few drops of Tyrode's solution and cut with a razor blade into pieces about 1 mm. in diameter. The marrow from each pig was divided into equal numbers of pieces for each solution under test and placed in separate dishes containing Tyrode's solution. It was from these dishes that the bone marrow was distributed among the culture dishes. Eye droppers with slightly enlarged openings were used for lifting the pieces of bone marrow. Care was taken to use only regularly shaped pieces. Any ragged ones were discarded. Six pieces of bone marrow were placed in each dish containing the liver dilution. The serum solution was warmed at 45°C. and mixed with the agar solution at 45°C. in the ratio of three parts of serum to five parts of agar. Care was taken to avoid overheating the serum. One cc. of this mixture was put in each of the dishes and mixed with the liver extract solution by swirling the dish. The pieces of bone marrow were then arranged quickly in a symmetrical pattern in the dishes with the aid of a thin blunt glass rod. Tops were placed on the dishes, and when the medium had set (usually a matter of seconds) the dishes were placed in the incubator at 37°C. for $4\frac{1}{2}$ to 5 hours.

When the incubation time had elapsed, the dishes were placed in a projector which magnified the bone marrow and its area of migration at least tenfold. The images of the explant and its area of migration were traced on squared paper and the areas measured by counting squares. The difference in area of these two was taken as the area of migration and was used in all calculations. All results were tested statistically for a significant difference between the points and a significant slope and deviation from linearity.

RESULTS. Table 1 shows the results of a typical test. This test was done using clinically active liver extract Lot No. 119 (Connaught Medical Research Laboratories 15 units per cc.). Table 2 shows the analysis of variance of these results.

Figure 1 shows the results of tests done on clinically active extracts and figure 2, shows the results of those done on clinically inactive extracts. All migrations shown are averages of the migrations obtained at those dilutions in the number of tests indicated in table 3. Those tests not averaged for the graphs were done at different dilutions and therefore could not be included. Table 3 is a summary of all the results obtained using this method of testing.

From these results it appears that there is a substance present in clinically active liver extracts which influences the migration of leucocytes from explants of guinea pig bone marrow, and which may be measured in some degree by the area of migration produced.

TABLE 1
Results of test no. 70 on clinically active lot no. 119

DILUTION	AREAS OF MIGRATION (THE NUMBER OF 4MM ² SQUARES ON GRAPH PAPER)												TOTAL
0:001	43	60	42	65	107	55	76	96	156	62	73	77	912
0:004	117	126	136	107	113	101	96	134	99	132	98	115	1,374
0:007	140	127	74	91	140	162	96	154	90	137	145	119	1,475
0:010	124	150	134	125	134	145	225	190	141	222	245	189	2,024
Total..												5,785

TABLE 2
Analysis of variance for test no. 70

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	VARIANCE	F
Doses	52105	3	17368	18*
Error	42385	44	963	
Total	94490	47		
Linearity				51*
Deviation from linearity				1.5

* Significant above the 1% level

DISCUSSION. During the course of this work it became evident that growing guinea pigs, received at about 200 grams and kept on ordinary rations supplemented by about 5 mg. of ascorbic acid given orally per day for about 3 weeks, were the most desirable for this work. Irregularities in the tests, poor migration, and sometimes negative results we believe to be in some part due to guinea pig variation. The other variable, the variation due to the plasma, which was present previously has been overcome to some extent by substitution of the agar-serum mixture. This more constant culture medium tends to give greater uniformity and reproducibility of results. It also has the advantage of simplifying the procedure.

Some difficulty has been encountered lately with the agar. Most of the tests reported were done using a pre-war stock. When a new stock of Difco agar was obtained the gel formed by this agar at the same concentration as formerly used

was found to be much too firm for good migration and it was necessary to experiment with different concentrations to obtain a suitable migration medium.

In determining the activity of a solution of liver extract at least three tests were done. Only in cases where there was a significant difference between the points and where the test for slope was significant (at the 5% level) were the

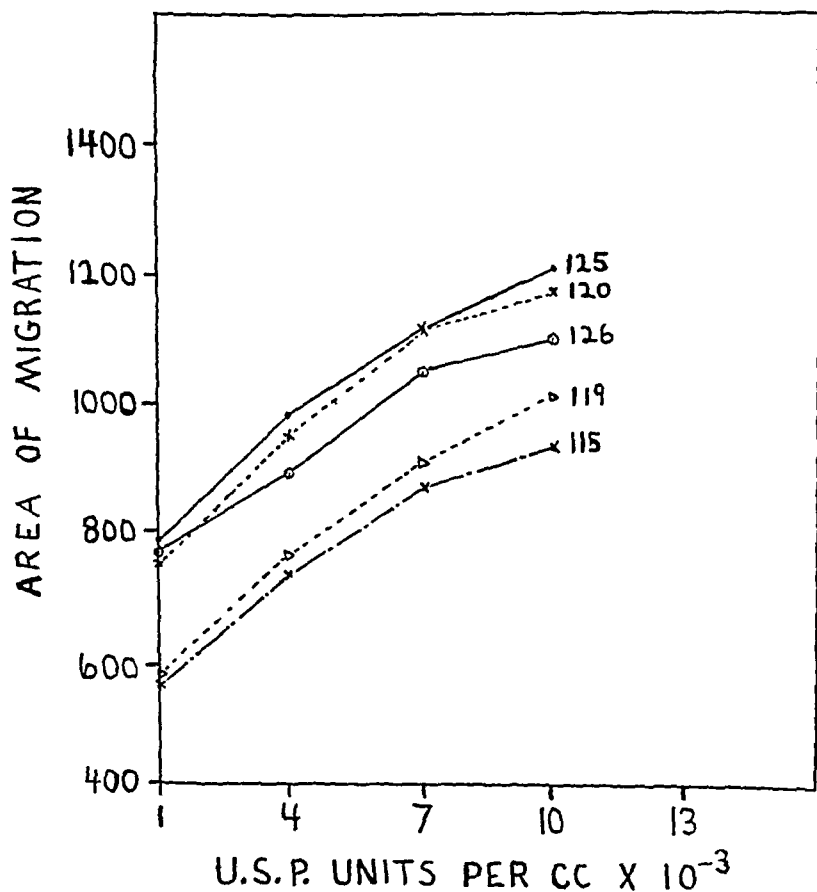


FIG. 1. THE RESPONSE OF GUINEA PIG BONE MARROW TO CLINICALLY ACTIVE EXTRACTS OF LIVER

results considered positive. In some cases the departure from linearity was also significant. In every test a control test consisting of 4 dilutions of a known active extract was set up using marrow from the same guinea pigs and the same basic culture medium for both the control solution and the unknown extract. If the control solution failed to give a statistically significant slope in the test then any result obtained in that test on the unknown solution was discarded.

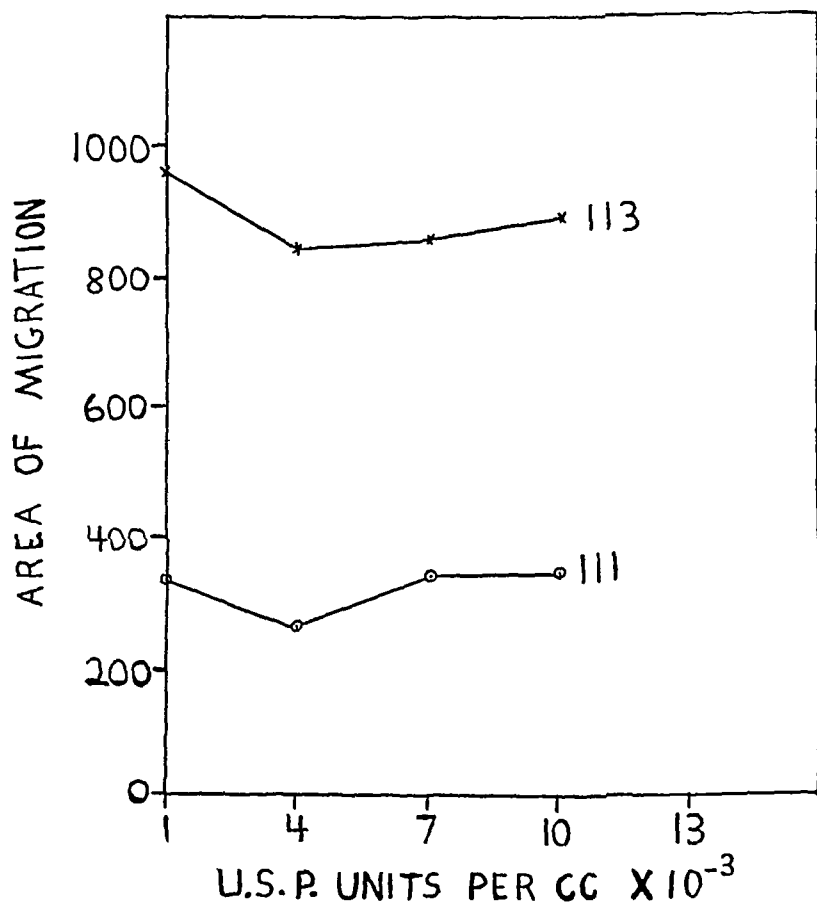


FIG. 2. THE RESPONSE OF GUINEA PIG BONE MARROW TO CLINICALLY INACTIVE EXTRACTS OF LIVER

TABLE 3
Results of tests

LOT NO.	CLINICAL ACTIVITY	NO. OF TESTS	NO. SIGNIFICANT	NO. AVERAGED FOR FIGURES 1 & 2
111	inactive	3	0	3
113	inactive	4	0	4
115	active	17	14	10
119	active	12	11	7
120	active	6	6	5
125	active	2	2	2
126	active	6	6	6

From the results obtained, and until it can be shown that a clinically active solution does not give the response described, it would appear that this test may be useful in determining whether or not liver extract has anti-pernicious anemia activity.

In view of the recent reports of the anti-pernicious anemia activity of folic acid (15, 16, 17), the reaction of this compound in the bone marrow test is of interest. Preliminary experiments indicate that folic acid (synthetic L. casei factor) is active in this test although the concentration required to produce the same response as active liver extract is considerably higher than that reported for liver extracts (18). This would indicate either than the concentration of folic acid reported for liver extracts is too low or that folic acid is not the only substance in the extracts which will produce the response. Xanthopterin also produces a similar response but at slightly higher concentrations than folic acid.

SUMMARY

The migration of cells from explants of guinea pig bone marrow has been studied. This migration appears to be controlled by the addition of extracts containing the anti-pernicious anemia substance. In 90 per cent of the tests, the relationship between concentration and migration could be expressed as a linear function. This test may be useful in the qualitative testing of liver extracts for anti-pernicious anemia activity.

This work was under the supervision of Dr. E. W. McHenry. Many of the assays were carried out by Miss Margaret Bell. To these and other members of the Staff of the Connaught Medical Research Laboratories who contributed to these experiments we wish to extend our thanks.

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THE INFLUENCE OF CERTAIN SULFHYDRYL COMPOUNDS ON THE TOXICITY OF AN ORGANIC MERCURIAL DIURETIC

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A total of forty cases (1, 2, 3, 4) of sudden death following the intravenous injection of organic mercurial diuretics in patients has been reported since Redlich (5) described the first three accidents of this type in 1925.

Such fatalities appear to be rare. They have, however, stimulated several investigations on the acute toxicity of mercury compounds given intravenously to animals as well as a search for preventive measures. Organic and inorganic mercury compounds, injected intravenously in adequate quantity in intact cats and dogs, have been found to produce a fall in blood pressure and a rise in venous pressure (6, 9, 10, 11). Electrocardiographic abnormalities accompanied and sometimes preceded these changes (2, 6, 7, 8). The more recent reports (2, 7, 8) agree that the death which occurs in animals shortly after the injection of various mercury compounds, including those used clinically for diuresis, results from a toxic action of mercury on the heart with resultant ventricular fibrillation and circulatory failure or respiratory failure secondary to the lowered blood pressure. On the basis of clinical observations, it has been suggested (1, 3, 4) that some of the deaths following the intravenous injection of organic mercurial diuretics in patients are due to this same mechanism.

Johnston (12) reported that "delerium cordis" produced by Salyrgan in the isolated turtle heart reverted to a regular rhythm when sodium thiosulfate was perfused through the heart along with the mercurial preparation, while DeGraff and Lehman (8) found that sodium thiosulfate had an antidotal action in the intact cat against toxic doses of mercurial compounds. Pines, et al. (2) noted that magnesium sulfate, when injected with esidrone, an organic mercurial diuretic, increased the amount of esidrone that could be injected intravenously in the dog before the appearance of ventricular fibrillation.

The present study was undertaken in order to test various substances containing sulfhydryl groups as possible antidotes to the cardio-toxic action of an organic mercury compound. The use of such substances was suggested by the recent reports on the effectiveness of 2,3 dimercaptopropanol (British Anti-Lewisite, BAL) in poisoning by various heavy metals (13, 14), by the reversal of the antibacterial action of inorganic mercury compounds with sulfhydryl compounds (15, 16), and by the reactivation of certain sulfhydryl-containing enzyme systems poisoned with p-chloromercuribenzoate by compounds containing —SH groups (17, 18).

¹ Supported by grants from Winthrop Chemical Company, Inc., New York, N. Y., and Schering Corporation, Bloomfield, New Jersey.

MATERIALS AND METHODS. The organic mercury compound employed was the diuretic Salyrgan² (without theophylline). This compound is the sodium salt of Salyrganic acid and contains 39.6 per cent mercury (19). A sterile 10 per cent stock solution in 2 cc. ampules was supplied by the manufacturer, and this preparation in appropriate dilutions in saline was used. Fresh 2.5 per cent solutions of 1 (+) cysteine hydrochloride, glutathione, and methionine³ were prepared in 0.9 per cent saline. A 0.2 per cent solution of 2,3-dimercaptopropanol⁴ was prepared in boiled saline. A 2.5 per cent solution of cystine was prepared in saline containing sufficient sodium hydroxide to dissolve the compound. The pH of the cystine solution was between 9 and 10.

The experiments on mice were conducted on male animals weighing 20 to 30 grams. Various doses of Salyrgan were given intravenously to groups of 10 mice; the percentage mortality and the 50 per cent lethal dose (that dose which should kill 50 per cent of a series of mice) were determined by the method of Behrens (20). The protective action of the sulfur-containing compounds was determined by giving a certain quantity of the material intravenously, then injecting Salyrgan one minute later by the same route. The extent of protection was evaluated from the increase of the 50 per cent lethal dose (LD₅₀) of Salyrgan. A total of 660 mice was used.

The action of Salyrgan on the circulation of dogs was investigated in 16 animals weighing between 5 and 15 kgm. They were anesthetized with Dial-Urethane (Ciba)⁵, 0.7 cc. per kgm., given intraperitoneally. Blood pressures were recorded from the left common carotid artery by means of a mercury manometer. Venous pressure records were obtained by introducing a long cannula into the superior vena cava through the right external jugular vein and connecting the cannula to a water manometer. Heart rates and electrocardiographic changes were recorded by means of a Grass ink-writing oscillograph. Lead 2 of the electrocardiogram was most frequently used. Salyrgan was injected either in repeated doses or by constant infusion into the right femoral vein. In the constant infusion experiments, the infusion of Salyrgan was continued throughout the experiment. After the appearance of toxic manifestations due to Salyrgan, the antidote to be studied was given through a long catheter or cannula inserted into the left external jugular vein as close as possible to the heart. In 9 experiments, the protective action of certain sulfhydryl-containing compounds was compared by giving a constant infusion of Salyrgan at a rate of 1.5 mgm. per kgm. per minute until definite toxic symptoms, as seen in the electrocardiogram and the venous and arterial pressures, appeared. A dose of the sulfhydryl-containing compound sufficient to cause the reversal of toxic symptoms was then injected intravenously. The period of time between the injection and the reappearance of the same degree of abnormality was considered to be the period of protection. In each experiment a dose of a sulfhydryl-containing compound was used as a standard and the compound to be tested was bracketed by the standard (s,x,s). In this way, 2,3-dimercaptopropanol, cysteine hydrochloride, and glutathione were compared. It was possible to make 3 to 5 injections in each animal. Attempts to give more than 5 injections failed because the blood pressure dropped to very low values. Thirty-three such injections were made in 9 dogs.

The studies on the isolated heart were conducted on 13 heart-lung preparations prepared according to Starling. The methods of preparation, recording, and testing the competence of the heart have been described in detail by Kraye (21) and by Kraye and Mendez (22). Two types of experiment were performed. In the first, repeated injections of Salyrgan were given into the venous supply reservoir. When cardiac failure had developed, the antidote was injected into the venous cannula as close as possible to the heart. In the second group, Salyrgan was given by continuous infusion throughout the experiment. When

² Kindly supplied by Winthrop Chemical Company, Inc., New York, N. Y.

³ Kindly supplied by Eli Lilly and Company, Indianapolis, Indiana.

⁴ Kindly supplied by Dr. E. S. Guzman Barron, The Chemical Division, Department of Medicine of the University of Chicago, Chicago, Illinois.

⁵ Kindly supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

toxic manifestations had developed, the SH-containing compound was given, and the interval between the injection of the sulfhydryl compound and the reappearance of cardiac failure was recorded.

RESULTS. *I. Influence upon the toxicity of Salyrgan in mice.* The intravenous LD 50 of Salyrgan was found to be 103 mgm. (0.204 millimols) per kgm. in the strain of mice used. The evidences of Salyrgan toxicity were characterized by a latency of 1 to 3 minutes followed by severe clonic and tonic convulsions which

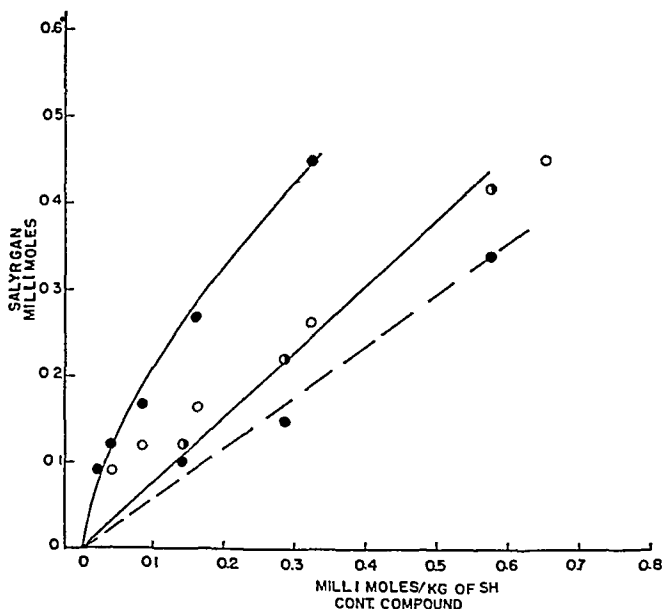


FIG. 1. THE PROTECTIVE ACTION OF CYSTEINE HYDROCHLORIDE, GLUTATHIONE, AND 2,3-DIMERCAPTOPROPANOL AGAINST SALYRGAN TOXICITY IN MICE

Ordinates: Increase in the intravenous L. D. 50 of Salyrgan in millimols. (L. D. 50 of Salyrgan with pretreatment minus L. D. 50 of Salyrgan without pretreatment.)

Abscissae: Millimols of —SH compounds injected. ●—●, 2,3-dimercaptopropanol in millimols. ○, 2,3-dimercaptopropanol in milliequivalents of —SH. ●—●, glutathione in millimols. ●---●, cysteine hydrochloride in millimols.

lasted 1 to 2 minutes and were terminated by the death of the animal. In the few animals in which convulsions did not lead to immediate death, paralysis of the hind legs appeared, and the animals died within 24 hours. In the series of 660 mice, no death later than the first 24 hours was observed, although all the animals were watched for at least a week after the injections.

The intravenous premedication of the mice with SH-containing compounds increased the LD 50 of Salyrgan. This increase was roughly proportional to the dose of the sulfhydryl compound administered (fig. 1). When the results are calculated on the basis of —SH equivalents, 2,3-dimercaptopropanol has

about the same protective action against Salyrgan as glutathione. The premedication with methionine and cystine also had a very weak protective action. Thus 0.286 millimols of methionine per kgm. increased the LD 50 of Salyrgan by 0.036 millimols per kgm., while 0.286 millimols of cystine per kgm. increased it by 0.067 millimols of Salyrgan per kgm. of body weight. Equivalent amounts (0.285 milliequivalents per kgm.) of 2,3-dimercaptopropanol, glutathione, and cysteine hydrochloride increased the L.D. 50 by 0.24, 0.22, and 0.15 millimols respectively.

II. *Influence on the circulatory failure caused by Salyrgan in anesthetized dogs.* The average lethal dose of mercury compounds for cats has been found by Modell and Krop (23) to be approximately 15 mgm. of mercury per kgm. DeGraff and Lehman (8) have found a value of 41 mgm. of Salyrgan (16.4 mgm. of mercury) per kgm. for the mean lethal dose in cats.

TABLE 1

The dose of Salyrgan in anesthetized dogs which produces electrocardiographic changes

Salyrgan was given by continuous infusion at a rate of 1.5 mgm./kgm./min. Anesthesia = 0.7 cc. per kg. Dial Urethane Ciba.

EXP. NO.	WEIGHT	EXPERIMENTAL TIME	TOXIC DOSE	
			Mgm. per kgm.	mM per kgm.
	kgm.	minutes		
1	8.7 F	18.5	27.56	0.0544
2	13.0 M	10.0	14.90	0.0294
3	7.5 F	15.5	23.10	0.0456
4	5.2 F	16.5	24.58	0.0486
5	6.7 F	13.0	19.37	0.0383
6	5.9 M	22.0	32.78	0.0647
7	7.0 M	13.5	20.12	0.0397
Average		15.6	23.20	0.0458
Standard error		± 1.23	± 2.15	± 0.0042

In the present study, the average dose of Salyrgan which produced definite electrocardiographic changes in dogs was 23.2 mgm. (9.19 mgm. of mercury) per kgm. of body weight (table 1). In all animals killed by Salyrgan, the cause of death was ventricular fibrillation. In the experiment illustrated by figure 2, a constant infusion of Salyrgan was given throughout the experiment at a rate of 3.7 mgm. per kgm. per minute. After 5 to 6 minutes, a definite rise in venous pressure and a fall in arterial pressure occurred which rapidly progressed to a severe impairment of the circulation, characterized by a marked fall in blood pressure, a concomitant rise in venous pressure, and electrocardiographic changes (fig. 3). All these effects were promptly corrected by an intravenous injection of 100 mgm. of cysteine hydrochloride. The cysteine hydrochloride injected was sufficient to protect the heart against a continuing infusion of 44.4 mgm. of Salyrgan, after which cardiac irregularities, fall in arterial pressure, and

rise in venous pressure reappeared, followed by ventricular fibrillation, which resulted in the death of the animal (fig. 2). Figure 3 represents the electrocardiographic tracing taken during the experiment represented by figure 2. The severe toxic manifestations produced by Salyrgan could be rapidly counteracted by 100 mgm. of cysteine hydrochloride. In a similar manner, it has been possible to treat severe toxic manifestations of Salyrgan poisoning with equivalent amounts of glutathione and 2,3,-dimercaptopropanol. The most severe toxic manifestations short of ventricular fibrillation could be completely reversed by adequate doses of the SH— containing compounds. Once ventricular fibrillation had begun, however, the administration of none of these compounds was effective.

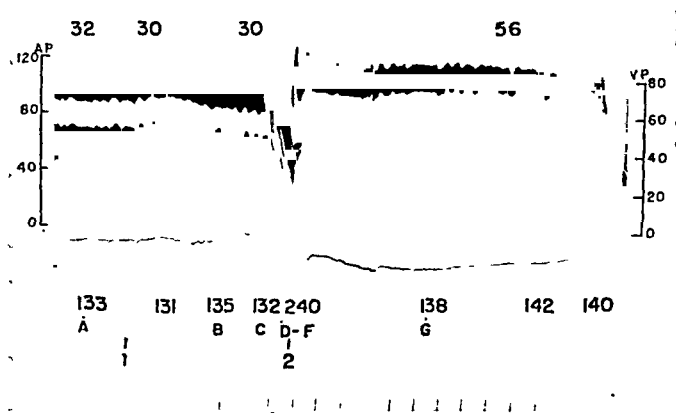


FIG. 2. THE EFFECT OF SALYRGAN AND CYSTEINE HYDROCHLORIDE ON THE ARTERIAL AND VENOUS PRESSURE IN THE INTACT ANESTHETIZED DOG

Female dog, 7.5 kgm., under Dial-Urethane, 0.7 cc. per kgm. intraperitoneally. Time in minutes. Figures from top to bottom: respiration rate, heart rate.

Letters A-G, electrocardiographic tracings corresponding to the letters in figure 3. Tracings from top to bottom: arterial pressure, central venous pressure.

1. Start of constant infusion of Salyrgan at a rate of 3.7 mgm. per kgm. per min. which continued all through the experiment

2. 100 mgm. cysteine hydrochloride intravenously.

No improvement in circulatory function was observed with methionine and cysteine, even though the constant infusion of Salyrgan was discontinued.

The results of the comparison of the protective action of 2,3,-dimercaptopropanol, glutathione, and cysteine hydrochloride are summarized in figure 4. 2,3,-dimercaptopropanol in equivalent amounts (of —SH groups) is far more effective than either glutathione or cysteine hydrochloride.

III. Influence upon heart failure caused by Salyrgan in the isolated mammalian heart. Toxic manifestations of Salyrgan in the heart-lung preparation, as in the intact animal, were mainly referable to the heart. Lung damage was relatively much less pronounced, and in no case was early lung edema observed. Single repeated injections or constant infusions were given into the heart-lung prepara-

tions. Table 2 shows the concentrations in millimols of Salyrgan per kgm. of heart-lung preparation (blood, heart, and lungs) which produced mild, medium, or severe heart failure. Mild heart failure was characterized by a slight increase in the right and left atrial pressure and a reduction of less than 10 per cent in systemic output. Medium failure was considered to be present if there was a definite rise in the atrial pressure, a rise in the pulmonary arterial pressure and a

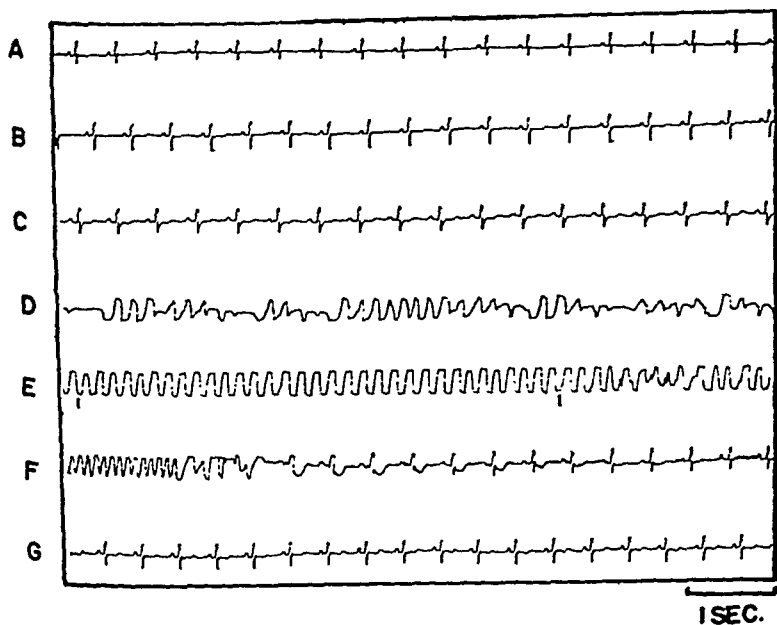


FIG. 3. THE EFFECT OF SALYRGAN AND CYSTEINE HYDROCHLORIDE ON THE ELECTROCARDIOGRAM OF THE INTACT ANESTHETIZED DOG. SAME EXPERIMENT AS THAT OF FIG. 2

- A. 2 minutes before continuous infusion of Salyrgan was started.
- B. 4 minutes after start of continuous infusion of Salyrgan.
- C. 2 minutes after B.
- D. 1.5 minutes after C.
- E. 0.5 minutes after D. Between the two marks, 100 mgm. of cysteine hydrochloride given intravenously.
- F. 10 seconds after the end of E.
- G. 5 minutes after F.

fall of the systemic output to about 50 per cent of normal. Severe failure was characterized by maximal rise in right and left auricular pressures and asystolic output less than 25 per cent of normal. Coronary sinus outflow (4 experiments) was not significantly changed by administration of Salyrgan until severe cardiac failure was induced, when it was reduced concomitantly with the decrease in arterial pressure. The drop in inflow pressure satisfactorily explains the change

in coronary sinus outflow. It can be seen from table 2 and figure 5 that about two-thirds of the dose of Salyrgan producing a severe heart failure did not pro-

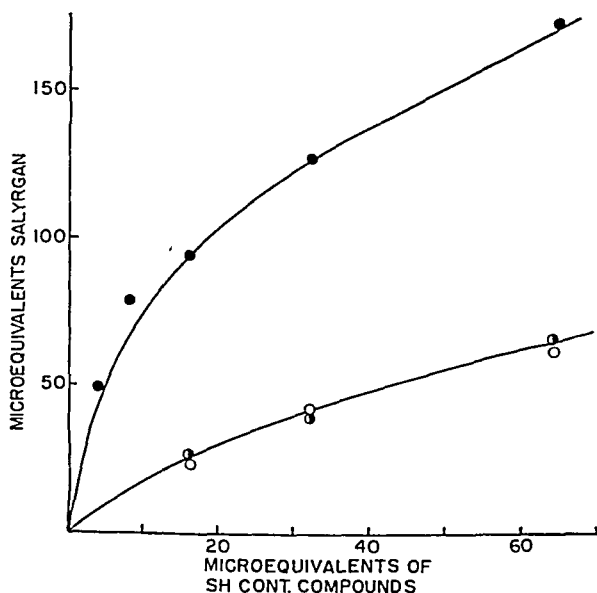


FIG. 4. THE PROTECTIVE ACTION OF GLUTATHIONE, CYSTEINE HYDROCHLORIDE, AND 2,3-DIMERCAPTOPROPANOL AGAINST SALYRGAN TOXICITY IN THE INTACT ANESTHETIZED DOG

Ordinate: Microequivalents of Salyrgan counteracted.

Abscissae: Microequivalents of —SH injected. ●—●, 2,3-dimercaptopropanol. ○—○, cysteine hydrochloride. ●—●, glutathione.

TABLE 2

The influence of various concentrations of Salyrgan on the heart-lung preparation of the dog

Data collected from 12 experiments. Blood Temperature, 37.5–39.1-C. Resistance, 75–80 mm Hg.

DEGREE OF HEART FAILURE	NUMBER OF OBSERVATIONS	mM OF SALYRGAN/LITER OF BLOOD		
		Average	Maximum	Minimum
Mild	4	0.274	0.438	0.159
Medium	7	0.334	0.485	0.233
Severe	7	0.384	0.547	0.291

duce any detectable changes in the activity of the heart. The dose which produced maximum failure was approximately one and a half times that which produced mild failure. With other cardiotoxic substances, such as Atabrine, sodium

pentobarbital, cocaine, and procaine, three to four times the minimal negative inotropic dose is required to cause severe heart failure (24)

Some of the effects of Salyrgan presented in figure 5 could be interpreted as being due to changes in pulmonary vascular resistance. However, an increase in pulmonary resistance cannot cause the observed increase in the left auricular pressure. The change in the cardiac diastolic volume seen with doses of Salyrgan producing a medium degree of cardiac failure is accompanied by only a slight rise in pulmonary arterial pressure. Therefore, a considerable part of the enlargement of the heart, in the absence of a decrease in rate, must be due to a

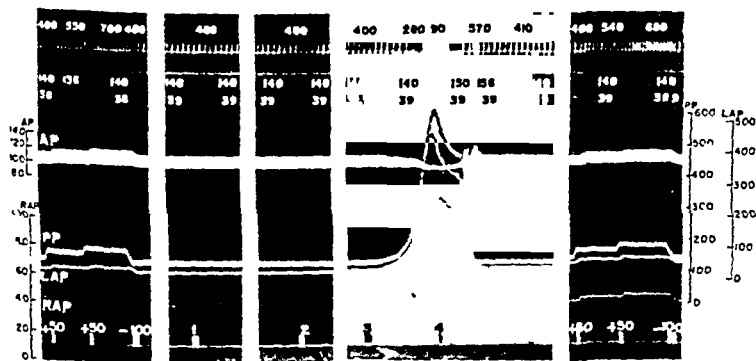


FIG 5. THE ACTION OF GLUTATHIONE ON CARDIAC FAILURE PRODUCED BY SALYRGAN IN THE HEART-LUNG PREPARATION OF THE DOG

Female dog, 10.7 kgm, under sodium pentobarbital, 35 mgm per kgm intraperitoneally. Arterial resistance, 72 mm of mercury. Ventricular weight, 78 grams. Figures from top to bottom: systemic output in cc per min, heart rate per min, blood temperature in degrees Centigrade. Tracings from top to bottom: systemic output, each signal indicating 100 cc, time in 10 sec intervals. A P, arterial pressure (A P scale in mm of mercury on left). P P, pulmonary arterial pressure (P P scale on right in mm of water). R A P, right auricular pressure (R A P scale on left in mm of water). L A P, left auricular pressure (L A P scale on right in mm of water).

+50, raising inflow reservoir by 50 mm

-100, lowering inflow reservoir to original level

At 1, 2, and 3, injection of 50 mgm of Salyrgan into venous reservoir, at 4, 100 mgm of glutathione

primary negative inotropic effect, rather than to an increase in pulmonary arterial resistance.

The administration of glutathione, cysteine hydrochloride, or 2,3,-dimecaptopropanol promptly reverses Salyrgan heart failure. A competence test before and after the administration of Salyrgan and glutathione showed that administration of the latter had restored the work capacity of the heart to normal (fig. 5). The influence of the sulfhydryl compound persists over a long period of time, provided the amount of Salyrgan in the heart-lung system is not too great. In the experiment of figure 5, for example, the effect persisted over 35 minutes; in another experiment, the effect of 2,3,-dimecaptopropanol lasted for over 80

minutes when it was terminated by giving further doses of Salyrgan. In the heart-lung preparation, as in the intact animal, it is possible to produce Salyrgan failure repeatedly, and to correct this failure again by further administrations of an SH—containing compound. However, while a single dose of Salyrgan can be counteracted completely, certain effects of subsequent doses are only temporarily reversed by the SH—containing compound (table 3). The cardiac irregularities caused by Salyrgan were less amenable to the therapeutic effect of the sulfhydryl-containing compounds than was the negative inotropic action.

In a manner similar to that used in the intact dog, the protective action against Salyrgan of 2,3,-dimercaptopropanol, cysteine hydrochloride, and glutathione

TABLE 3

The influence of repeated doses of Salyrgan and sulfhydryl compounds in the heart-lung preparation

Exp. 2. H.L.P., Female dog 10.6 kgm. Anesthesia, sodium pentobarbital. Weight of heart, 78 g. Arterial resistance, 72 mm Hg.

TIME	SUBSTANCE INJECTED	DOSE	PERIOD BETWEEN INJECTION AND REAPPEARANCE OF IRREGULARITIES	CARDIAC FAILURE	IRREGULARITIES OF CARDIAC RHYTHM
<i>min.</i>		<i>mgm.</i>			
0	Salyrgan	150	—	Severe	Severe
3	Glutathione	100	—	None	None
48	—	—	—	None	None
49	Salyrgan	100	—	Severe	Severe
59	Cysteine HCl	56	—	None	None
73.5	—	—	14.5	Slight	Severe
74	Cysteine HCl	56	—	None	None
85	—	—	11	Slight	Severe
94	Cysteine HCl	56	—	None	None
101.5	—	—	7.5	Slight	Severe
104	Cysteine HCl	56	—	None	None
109.5	—	—	5.5	Slight	Severe
117	Cysteine HCl	56	—	None	None
121.5	—	—	4.5	Slight	Severe
126	Glutathione	100	—	None	None
130.5	—	—	4.5	Slight	Severe

in the heart-lung preparation has been determined. The constant infusion of Salyrgan was continued all through the experiment. On the basis of an equivalent sulfhydryl group content, 2,3,-dimercaptopropanol had about the same activity in the heart-lung preparation as glutathione and cysteine hydrochloride (table 4), while in intact dogs, 2,3,-dimercaptopropanol was about 5 to 8 times as effective as either glutathione or cysteine hydrochloride (fig. 4). In no instance was there any definite improvement of Salyrgan failure with 200 milligram doses of cystine or methionine. In two heart-lung preparations, one with spontaneous and one with sodium pentobarbital failure, 2,3,-dimercaptopropanol, glutathione, and cysteine hydrochloride in doses of 10, 100, and 56 mgm. respectively had

only a negative inotropic effect on the heart; while 75 and 150 mgm. cystine and 45 and 90 mgm. of methionine had a slight but definite positive inotropic action in both types of experimental cardiac failure. In 3 heart-lung preparations, epinephrine hydrochloride (5 to 20 micrograms), veratridine hydrochloride (0.2 mgm.), and ouabain (0.1 mgm.) led to a definite improvement of Salyrgan heart failure.

DISCUSSION. It has been shown that the organic mercurial diuretic Salyrgan, when injected intravenously in adequate quantities produces severe impairment of myocardial function, accompanied by changes in the rhythm of the heart beat due to disturbances in impulse production and propagation. These severe effects occur with an average concentration of 3.8×10^{-4} M of Salyrgan. This concentration is of the same order as that found to inhibit certain SH—containing enzyme systems by p-chloromercuribenzoate (21, 22). The exact mechanism of this inactivation of Salyrgan by —SH compounds is not known. Data obtained with other mercury-containing compounds (21, 22) would make it seem likely that —SH

TABLE 4

The protective action of various SH-containing compounds against Salyrgan in the H.L.P. of the dog

Constant infusion of Salyrgan given at a rate of 6.5 mg per minute. Combined results from 4 heart-lung preparations.

	CYSTEINE HYDROCHLORIDE			GLUTATHIONE			2,3 DIMERCAPTOPROPANOL		
Microequivalents of SH compounds.	81	162	324	81	162	324	81	162	324
Micromols of Salyrgan counteracted.....	76.5	101.3	167.5	80	103.4	192.7	77.6	119.6	209.1
Number of determinations.....	4	2	1	3	2	1	2	6	2

compounds inactivate Salyrgan through formation of a mercaptide linkage between mercury and the sulfhydryl group.

The amount of mercury inactivated in the intact dog is several times (with 2,3,-dimercaptopropanol up to 15 times) greater than could be explained on the basis of mercaptide formation. Mercury compounds, when given intravenously, rapidly disappear from the blood stream (25). Thus it is conceivable that the diffusion of Salyrgan from the circulation into organs like liver, muscles, and brain may markedly reduce the amount of Salyrgan which has to be inactivated by the sulfhydryl compounds in the blood stream in order to protect the heart against Salyrgan. In the heart-lung preparation, sulfhydryl compounds counteracted Salyrgan in approximately equivalent amounts when small doses of —SH compounds were used and in less than equivalent amounts when large doses were given. In the isolated heart and lungs, little tissue is available into which the Salyrgan can diffuse, and therefore most of the Salyrgan remains accessible to inactivation by the sulfhydryl compounds in the blood stream.

In intact dogs, 2,3,-dimercaptopropanol is approximately 5 to 8 times more potent than glutathione or cysteine hydrochloride, while in the heart-lung preparation of the dog, equivalent amounts of the three substances are about equally effective in protecting the heart against Salyrgan (table 5). This difference may be due to a different rate of oxidation of the $-SH$ compounds in the two systems or to a slower rate of diffusion of 2,3,-dimercaptopropanol than of glutathione and cysteine hydrochloride from the blood stream to other organs.

In mice, Salyrgan was inactivated by approximately one equivalent of sulfhydryl compound, and no differences in the protective action of the three sulfhydryl compounds studied were observed. The very short time between the injection of Salyrgan and the $-SH$ compound may have minimized the effects due to oxidation and diffusion of the $-SH$ compound.

SUMMARY

The organic mercurial compound used in this study produced toxic manifestations which were referable mainly to the heart when it was given intravenously to animals in large enough dosage. These effects were counteracted by the intravenous injection of the sulfhydryl compounds cysteine hydrochloride, glutathione, and 2,3,-dimercaptopropanol. In the heart-lung preparation of the dog, the protective action of the three sulfhydryl compounds studied was about equal when calculated in terms of $-SH$ equivalents, while in the intact dog, 2,3,-dimercaptopropanol was about 5 to 8 times as effective as either cysteine hydrochloride or glutathione. Cystine and methionine had a slight protective action against the organic mercury compound in mice, but none in the intact dog or in the heart-lung preparation of the dog. Cysteine hydrochloride, glutathione, and 2,3,-dimercaptopropanol did not improve either spontaneous or sodium pentobarbital cardiac failure in the dog heart-lung preparation.

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THE TOXICITY AND TOXIC MANIFESTATIONS OF 2,2-BIS-(p-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE (DDT) AS INFLUENCED BY CHEMICAL CHANGES IN THE MOLECULE

A CONTRIBUTION TO THE RELATION BETWEEN CHEMICAL CONSTITUTION AND TOXICOLOGICAL ACTION

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Few chemicals have aroused as much interest as 2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane (DDT) which, although synthesized by Zeidler (1) as early as 1874 has gained enormous interest only recently by the discovery of its marked insecticidal action by Müller (2). The toxicity of this material for mammals by various routes of administration has been studied by various investigators (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14). It has been shown that with oral and cutaneous administration as well as with inhalation there is a considerable variation in the susceptibility of different species. In our experience, mice were found to be the most sensitive experimental animals. The manifestations of sub-fatal and fatal doses of DDT are hyperexcitability, tremors, and convulsions, and with fatal doses these are followed by coma and death. These symptoms are readily observed and it was thought these could be used as a tool in studying the effects of changes in the DDT molecule on the toxicity for mice and on certain physical and chemical properties such as the solubility in oil, the rate of dehydrochlorination and others.

In addition to DDT (No. 1),¹ 22 derivatives of DDT were studied in these experiments, as follows:

1. 2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane
2. 2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene

¹ The DDT (No. 1) used in these experiments was a purified, recrystallized material which, like numbers 4, 7, 11, 14, and 23, was kindly furnished to us by Dr. H. L. Haller from the Beltsville Experimental Station of the U. S. Department of Agriculture; number 13 was given to us by Prof. M. S. Newman, Ohio State University, Columbus, Ohio; and number 3 was received through Dr. R. A. Ormsbee, Insect Control Committee, National Research Council, Washington, D. C.; to all of whom we owe many thanks for their kindness. The other compounds were synthesized in our laboratory according to the existing literature, namely number 5 according to Montagne (*Rec. trav. chim.*, 25: 379, 1906); numbers 8, 10 and 22 according to Zeidler (*Ber. deut. chem. Ges.*, 7: 1180, 1874); numbers 15 and 16 according to ter Meer (*Ber. deut. chem. Ges.*, 7: 1200, 1874); numbers 18 and 19 according to Fritsch and Friedmann (*Annal.*, 306: 72, 1899); numbers 20 and 21 according to Prill, Hartzell, and Arthur (*Science*, 101: 464, 1945); numbers 2 and 6 according to White and Sweeney (*Pub. Health Rep.*, 60: 66, 1945); number 9 according to Chattaway (*J. Chem. Soc.*, 701, 1934); number 12 according to Baeyer (*Ber. deut. chem. Ges.*, 7: 1190, 1874); and number 17 according to Fischer (*Ber. deut. chem. Ges.*, 7: 1191, 1874).

3. 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane
4. 2,2-bis-(p-chlorophenyl)-1-monochloroethane
5. di-(p-chlorophenyl)-methane
6. di-(p-chlorophenyl)-acetic acid
7. 2,2-bis-(p-chlorophenyl)-1,1,1-tribromoethane
8. 2,2-bis-(p-bromophenyl)-1,1,1-trichloroethane
9. 2,2-bis-(p-iodophenyl)-1,1,1-trichloroethane
10. 2,2-bis-(p-fluorophenyl)-1,1,1-trichloroethane
11. 2-(p-chlorophenyl)-2-phenyl-1,1,1-trichloroethane
12. 2,2-bis-phenyl-1,1,1-trichloroethane
13. 2,2-bis-(m,p-chlorophenyl)-1,1,1-trichloroethane
14. 2,2-bis-(o,p-chlorophenyl)-1,1,1-trichloroethane
15. 2,2-bis-(hydroxyphenyl)-1,1,1-trichloroethane
16. 2,2-bis-(p-acetoxyphenyl)-1,1,1-trichloroethane
17. 2,2-bis-(p-anisyl)-1,1,1-trichloroethane
18. 2,2-bis-(p-phenetyl)-1,1,1-trichloroethane
19. 2,2-bis-(p-propoxyphenyl)-1,1,1-trichloroethane
20. 2,2-bis-(p-butoxyphenyl)-1,1,1-trichloroethane
21. 2,2-bis-(p-amyloxyphenyl)-1,1,1-trichloroethane
22. 2,2-bis-(p-tolyl)-1,1,1-trichloroethane
23. 2,2-bis-(p-tert.-butylphenyl)-1,1,1-trichloroethane

EXPERIMENTAL TECHNIQUE. The toxicity of these compounds was determined by giving them orally to mice as 5, 10 or 25 per cent solutions in olive oil, or in a few instances as a 10 per cent solution in 20 or 25 per cent acetone in olive oil, the concentration used depending upon the solubility of the material and its toxicity in order to avoid the administration of abnormally large or abnormally small amounts of the solutions. Only di-(p-chlorophenyl)-acetic acid was given as sodium salt in aqueous solution. The calculated doses of these solutions were introduced into the stomach through the esophagus by means of a tuberculin syringe with a blunt needle. With few exceptions male white mice of 20-30 gm. body weight were used for these experiments and generally groups of 10 animals were fed with each dose. The mice were kept in individual cages and were fed Purina dog chow and water. They were observed for one week and the incidence of tremors, convulsions, and fatalities was noted.

The rate of the *dehydrochlorination*, the splitting off of hydrochloric acid with the formation of dichloroethylene derivatives, was determined by the same procedure as described by Cristol (15), making the determinations at 37°C. instead of 20.11°C. and 30.37°C. as used by this author.

The *solubility in oil* was determined by adding, from a weighed amount of the chemicals, small increments to 1 cc. of olive oil kept at 37°C. until no further material went in solution after agitation, and determining the amount of the material required for this purpose by weighing back the remaining amount.

RESULTS. *I. Effect of changes in the trichloroethane group of DDT.* As illustrated in table 1, the *toxicity* of DDT and especially its characteristic ability to cause tremors and convulsions is bound to the presence of the trichloroethane group. Splitting off one molecule of hydrochloric acid, resulting in the formation of 2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethylene, decreases the toxicity of DDT from a L.D.₅₀ of 200 mg./kg. to about 700 mg./kg. and abolishes the charac-

teristic tremors. Saturation of the double bond in the dichloroethylene derivative, as in 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane, decreases the toxicity further to above 1000 mg./kg. and does not reestablish the ability to cause tremors and convulsions; and the same holds true for the lower homologue, 2,2-bis-(p-chlorophenyl)-1-monochloroethane. The two chlorine-free derivatives, di-(p-chlorophenyl)-methane and di-(p-chlorophenyl)-acetic acid, cause no tremors and the L.D.₅₀ of the former is higher than 2000 mg./kg. and that of the latter is about 700 mg./kg. Replacement of all 3 chlorine atoms of the trichloroethane group by bromine, as in 2,2-bis-(p-chlorophenyl)-1,1,1-tribromoethane.

TABLE I
EFFECT OF CHANGES IN THE TRI-CHLOROETHANE GROUP ON THE TOXICITY AND PHYSICAL-CHEMICAL PROPERTIES OF
2,2-BIS-(P-CHLOROPHENYL)-1,1-TRI-CHLOROETHANE

NAME	2,2-BIS-(P-CHLORO- PHENYL)-1,1-TRI- CHLOROETHANE	2,2-BIS-(P-CHLORO- PHENYL)-1,1-DI- CHLOROETHYLENE	2,2-BIS-(P-CHLORO- PHENYL)-1,1-DI- CHLOROETHANE	2,2-BIS-(P-CHLORO- PHENYL)-1-CHLORO- ETHANE	DI-(P-CHLORO- PHENYL)-METHANE	2,2-BIS-(P-CHLORO- PHENYL)-ACETIC ACID	2,2-BIS-(P-CHLORO- PHENYL)-1,1,1-TRI- BROMOETHANE		
FORMULA									
MOL. WEIGHT	354.49	318.03	320.05	285.60	237.12	280.04	482.87		
MELT. POINT °C	106.5-109.0	68-69	109.4-110.2	51-53	55	165-166	143-146		
SOLUBILITY	INSOL. ALCOHOL OLIVE OIL	INSOL. SOL. 20 %	INSOL. SOL. 8.0 %	INSOL. SOL. —	INSOL. SOL. —	INSOL. SOL. —	INSOL. SOL. 2.0 %		
INCIDENCE OF DEATH									
SOLVENT	OLIVE OIL	OLIVE OIL	OLIVE OIL	OLIVE OIL	OLIVE OIL	WATER (100:50:50)	OLIVE OIL		
CONCENTRATION	10 %	10 %	10 %	10 %	20 %	20 %	10 %		
	NO. OF ANIMALS	% DEATH	NO. OF ANIMALS	% DEATH	NO. OF ANIMALS	% DEATH	NO. OF ANIMALS	% DEATH	
DOSE IN MG./KG.	75	5	20						
	100	15	7	10	0	10	0	10	0
	150	15	14						
	200	15	87	10	10	10	0	10	0
	300	15	66	10	30	10	0	4	0
	400	25	70	13	30	10	0	10	0
	500	15	80	7	29	8	0	10	10
	600			11	55	10	20	10	30
	700			11	55	10	0	10	40
	800			10	50	10	0	10	0
	900			10	60	10	0	10	0
	1000			10	100	8	0	10	0
1500									
2000									
TREMORS	YES	NO	NO	NO	NO	NO	NO	NO	
DEHYDROCHLORINATION RATE	12,56	0	4,035	563 (CALC.)	0	0	ca 127,000		

increases the L.D.₅₀ to more than 700 mg./kg. and abolishes the nervous manifestations of DDT.









As illustrated in table 1, the rate of dehydrochlorination decreases from 12,515 for DDT over 4,035 for 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane to 563 for monochloroethane, whereas 2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene does not split off hydrochloric acid, presumably because of the double bond. The tribromoethane derivative splits off hydrobromic acid very easily ($K = \text{ca } 127,000$) which is in accordance with its great instability towards heating and solvents.

As illustrated in table 1, the solubility in olive oil is considerably increased by

dehydrochlorination, the solubility of DDT being 10.5 per cent and that of 2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene being 21.1 per cent. On the other hand, the solubility of 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane is of a similar order as that of DDT, namely 8.0 per cent, and that of 2,2-bis-(p-chlorophenyl)-1,1,1-tribromoethane only 2 per cent.

II. Effect of replacing chlorine in the p-chlorophenyl group by bromine, iodine, fluorine and hydrogen and of changes of the position of the chlorine atom in the phenyl ring. As illustrated in table 2, neither the toxicity of DDT nor its effect on the nervous system are changed by replacing chlorine in the phenyl ring by bromine, as in 2,2-bis-(p-bromophenyl)-1,1,1-trichloroethane, the L.D.₅₀ of

TABLE 2
EFFECT OF REPLACING CHLORINE IN THE CHLOROPHENYL GROUP BY BROMINE, IODINE, FLUORINE, AND HYDROGEN AND CHANGES OF THE POSITION OF THE CHLORINE ATOM IN THE PHENYL GROUP ON TOXICITY AND PHYSICAL-CHEMICAL PROPERTIES OF 2,2-BIS-(P-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE









NAME	2,2-BIS-(P-CHLORO- PHENYL)-1,1-TRI- CHLOROETHANE	2,2-BIS-(P-BROMO- PHENYL)-1,1-TRI- CHLOROETHANE	2,2-BIS-(P-IODO- PHENYL)-1,1-TRI- CHLOROETHANE	2,2-BIS-(P-FLUORO- PHENYL)-1,1-TRI- CHLOROETHANE	2,2-BIS-(P-CHLORO- PHENYL)-1,1-TRI- CHLOROETHANE	2,2-BIS-(P-CHLORO- PHENYL)-1,1-TRI- CHLOROETHANE	2,2-BIS-(P-CHLORO- PHENYL)-1,1-TRI- CHLOROETHANE	2,2-BIS-(P-CHLORO- PHENYL)-1,1-TRI- CHLOROETHANE
FORMULA								
MEL. POINT °C	254.49	443.8	537.42	321.58	320.05	285.60	354.49	354.49
MEL. POINT °C	103-109	129-136	172 (194)	42-45	770-775	64	100	740-745
SOLUBILITY	WATER ALCOHOL GLYCE OL	INSOL SOL 2%	INSOL SOL 15%	INSOL SOL 745%	INSOL SOL 228%	INSOL SOL 33.7%	INSOL SOL —	INSOL SOL —
INCIDENCE OF DEATH								
SOLVENT	OLIVE OIL	OLIVE OIL	OLIVE OIL	OLIVE OIL	OLIVE OIL	OLIVE OIL	OLIVE OIL	OLIVE OIL
CONCENTRATION	10 %	5 %	5 %	10 %	10 %	10 %	10 %	10 %
	NO. OF ANIMALS	% DEATH	NO. OF ANIMALS	% DEATH	NO. OF ANIMALS	% DEATH	NO. OF ANIMALS	% DEATH
DOSE IN MG/KG	100 200 300 400 500 600 700 800 900 1000	13 15 15 25 25 80 — — — —	7 10 10 10 10 70 80 — — —	10 10 10 10 10 10 10 10 10 10	0 0 0 0 0 0 0 0 0 0	10 10 10 10 10 10 10 10 10 10	0 0 0 20 0 20 30 20 10 10	10 10 10 10 10 10 10 10 10 10
TREMORS	YES	YES	YES	YES (SLIGHT)	NO	NO	NO	NO
CONVULSION RATE	12,515	10,760	19,800	2,319	2,200	272	16,300	255

both compounds being 200 mg./kg. Both are very little affected by substitution of iodine, the L.D.₅₀ of 2,2-bis-(p-iodophenyl)-1,1,1-trichloroethane being 400 mg./kg., but they are affected more by the introduction of fluorine, as in 2,2-bis-(p-fluorophenyl)-1,1,1-trichloroethane, the L.D.₅₀ of which is about 900 mg./kg. Replacing one or both chlorine atoms in the phenyl ring by hydrogen, as in 2-(p-chlorophenyl)-2-phenyl-1,1,1-trichloroethane and 2,2-bis-phenyl-1,1,1-trichloroethane, reduces the toxicity considerably, the L.D.₅₀ of the former being greater than 1000 mg./kg. and of the latter above 800 mg./kg., the largest doses which could be given conveniently, both compounds causing neither tremors nor convulsions. The same holds true if the chlorine atom in one phenyl ring is shifted to ortho or meta position as in 2,2-(m,p-chlorophenyl)-1,1,1-trichloro-

ethane and in 2,2-(o,p-chlorophenyl)-1,1,1-trichloroethane. As illustrated in table 2, the rate of *dehydrochlorination* of the p-chlorophenyl, p-bromophenyl and p-iodophenyl derivatives is of the same order, namely 12,515, 18,760 and 19,800, whereas that of the p-fluorophenyl derivative is 2,319, that of 2-(p-chlorophenyl)-2-phenyl-1,1,1-trichloroethane is 2,200, and that of 2,2-bis-phenyl-1,1,1-trichloroethane is only 272. The o,p-chlorophenyl derivative had also a low rate of dehydrochlorination, namely 255, whereas that of the m,p-chlorophenyl compound was of the high order of 16,300. It should, however, be pointed out that the latter was only available as a syrupy material and it is questionable to what extent this material was pure.

TABLE 2

EFFECT OF REPLACING CHLORINE IN THE PHENYL RING BY A HYDROXY GROUP AND OF ITS CLOSURE BY ESTERIFICATION OR ETHERIFICATION ON THE TOXICITY AND PHYSICAL-CHEMICAL PROPERTIES OF 2,2-BIS-(P-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE

NAME	2,2-BIS-(P-CHLORO-PHENYL)-W-TRI-CHLOROETHANE	2,2-BIS-(P-HYDROXY-PHENYL)-W-TRI-CHLOROETHANE	2,2-BIS-(P-ACETOXY-PHENYL)-W-TRI-CHLOROETHANE	2,2-BIS-(P-ANISYL)-W-TRI-CHLOROETHANE	2,2-BIS-(P-PHENOXY)-W-TRI-CHLOROETHANE	2,2-BIS-(P-PROPYLOXY)-W-TRI-CHLOROETHANE	2,2-BIS-(P-BUTOXY)-W-TRI-CHLOROETHANE	2,2-BIS-(P-PENTOXY)-W-TRI-CHLOROETHANE								
FORMULA																
MOL. WEIGHT	354.49	317.60	401.67	343.63	373.70	401.75	429.88	457.86								
MELT POINT °C	108.5-109.0	202 (recryst)	142-142.5	89	105 (subl-0.1)	62	50	62.5-70.5								
SOLUBILITY	WATER ALCOHOL OLIVE OIL	INSOL SL SOL 10.5%	INSOL SOL 0.9%	INSOL SOL 1.9%	INSOL SOL 3.7%	INSOL SOL 3.9%	INSOL SOL 32.5%	INSOL SOL 19.5%	INSOL SOL —							
INCIDENCE OF DEATH																
SOLVENT	OLIVE OIL		ETHYLACETONE-OLIVE OIL		OLIVE OIL		OLIVE OIL		OLIVE OIL		OLIVE OIL		OLIVE OIL			
CONCENTRATION	10 %		10 %		10 %		10 %		10 %		10 %		10 %			
	NQ.OF ANIMALS	% DEATH	NQ.OF ANIMALS	% DEATH	NQ.OF ANIMALS	% DEATH	NQ.OF ANIMALS	% DEATH	NQ.OF ANIMALS	% DEATH	NQ.OF ANIMALS	% DEATH	NQ.OF ANIMALS	% DEATH		
DOSE IN MG/KG	100	15	7	10	0	10	0	10	0	10	0	10	0	10	0	
200	15	15	10	0	10	0	10	0	10	0	10	0	10	0		
300	15	66	10	0	10	0	10	0	10	0	10	0	10	0		
400	25	70	10	0	10	0	10	0	10	0	10	0	10	0		
500	15	80			8	0	8	0	8	100	8	100	8	0		
600			8	40	10	0	10	0					10	0		
700					10	0	10	0					10	0		
800					10	0	10	20					10	0		
900					10	0	10	10					10	0		
1000					8	0							8	0		
TREMORS	YES		NO		NO		NO		YES		YES		NO		NO	
DEHYDROCHLORINATION RATE	12,515		24,70		50.8		76.8		433		620		591		732	

As illustrated in table 2, the *solubility in olive oil* is greatly reduced by replacing the chlorine atom in the phenyl ring by bromine and iodine to 2.0 and 0.5 per cent, respectively, whereas it is increased to above 45 per cent by the introduction of fluorine. The solubility in olive oil of 2-(p-chlorophenyl)-2-phenyl-1,1,1-trichloroethane and of 2,2-bis-phenyl-1,1,1-trichloroethane is of a similar order, namely 27.8 and 33.7 per cent whereas that of the m,p- and o,p-chlorophenyl derivatives could not be determined because of lack of material.

III. *Effect of replacing chlorine in the phenyl group by a hydroxy group and of its closure by esterification and etherification.** As illustrated in table 3, the *toxicity* of DDT is considerably reduced and its nervous manifestations are completely

abolished by replacing the chlorine atom in the phenyl ring by a hydroxylic group, as in 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, (L.D.₅₀ > 600 mg./kg.), and this is not reestablished by esterification with acetic acid, as in 2,2-bis-(p-acetoxyphenyl)-1,1,1-trichloroethane, (L.D.₅₀ > 1000 mg./kg.), nor by etherification with methanol, as in 2,2-bis-(p-anisyl)-1,1,1-trichloroethane, (L.D.₅₀ > 900 mg./kg.). On the other hand, etherification with ethanol and propanol, as in 2,2-bis-(phenetyl)-1,1,1-trichloroethane and 2,2-bis-(p-propoxyphenyl)-1,1,1-trichloroethane, forms compounds of the same toxicity and effect on the nervous system as DDT, the L.D.₅₀ for the two former being 300 and 200 mg./kg. as compared with 200 mg./kg. for DDT. In contrast to these compounds the two higher homologues, 2,2-bis-(butoxyphenyl)-1,1,1-trichloroethane and 2,2-bis-(p-amyloxyphenyl)-1,1,1-trichloroethane, are of a very low toxicity, the L.D.₅₀ being greater than 1000 mg./kg., and they are devoid of nervous manifestations.

As illustrated in table 3, the rate of dehydrochlorination of 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane is extremely small, being only about 7.0. Esterification of the hydroxylic group, as in 2,2-bis-(p-acetoxyphenyl)-1,1,1-trichloroethane, increases it to 50.8 and etherification with methanol, ethanol, propanol, butanol and amyl alcohol increases it to 76.8, 43.3, 62.0, 59.1 and 73.2, respectively.

As may be seen in table 3, the *solubility in olive oil* is very much reduced by replacing the chlorine atom by a hydroxylic group, the solubility of 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane being only 0.9 per cent as compared with 10.5 per cent for DDT. Esterification with acetic acid, as in 2,2-bis-(p-acetoxyphenyl)-1,1,1-trichloroethane, increased it slightly to 1.9 per cent. Etherification with methanol, as in 2,2-bis-(p-anisyl)-1,1,1-trichloroethane, and with ethanol, as in 2,2-bis-(p-phenetyl)-1,1,1-trichloroethane, increases it to 9.7 and 3.9, and etherification with propanol and butanol, as in 2,2-bis-(p-propoxyphenyl)-1,1,1-trichloroethane and 2,2-bis-(p-butoxyphenyl)-1,1,1-trichloroethane, increases it to 32.5 and 19.6 per cent, respectively. The solubility of the amyloxyphenyl derivative could not be determined because of lack of material.

IV. *Effect of replacing the chlorine atom in the phenyl ring by alkyl radicals.* As shown in table 4, the *toxicity* of DDT and its nervous manifestations are greatly reduced or abolished by replacing the chlorine atom in the phenyl ring by alkyl radicals such as a methyl or tertiary butyl group, the L.D.₅₀ of 2,2-bis-(p-tolyl)-1,1,1-trichloroethane being above 700 mg./kg. and that of 2,2-bis-(p-tertiary butylphenyl)-1,1,1-trichloroethane above 900 mg./kg., the highest doses which could be tested conveniently.

As illustrated in table 4, the *rate of dehydrochlorination* of these compounds is very low, being 75.6 for the tolyl derivative and 83.0 for the tertiary butyl derivative.

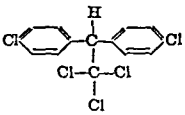
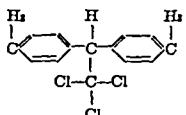
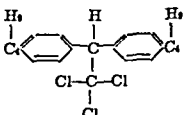
The *solubility in olive oil* could only be determined for the tolyl derivative for which is 12.1 per cent.

DISCUSSION OF THE EXPERIMENTAL FINDINGS. As illustrated in table 1 and

figure 1-A, the *toxicity and neurologic manifestations* of DDT in mice depend upon the presence of the trichloroethane group, and neither 2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene, 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane, 2,2-bis-(p-chlorophenyl)-1-monochloroethane, di-(p-chlorophenyl)-acetic acid nor di-(p-

TABLE 4

Effect of replacing chlorine in the phenyl ring by alkyl groups on toxicity and physical-chemical properties of 2,2-(p-chlorophenyl)-1,1,1-tri-chloroethane

NAME	2,2-BIS-(P-CHLORO-PHENYL)- 1,1,1- TRI-CHLOROETHANE	2,2-BIS-(P-TOLYL)-1,1,1-TRI- CHLOROETHANE	2,2-BIS-(P-tert.BUTYL-PHENYL)- 1,1,1-TRI-CHLOROETHANE
Formula			
Mol. weight.....	354.49	313.65	397.81
Melt point (°C.).....	103.5-109.0	88-89	154-155
Solubility			
Water.....	Insol.	Insol.	Insol.
Alcohol.....	Sl. sol.	Sol.	Sol.
Olive oil.....	10-5%	12.1%	—

Incidence of death

DOSE	SOLVENT					
	Olive oil		Olive oil		Olive oil	
	Concentration					
	10%		10%		10%	
	No. of animals	% death	No. of animals	% death	No. of animals	% death
mg./kg.						
100	15	7	10	0	10	0
200	15	47	7	0	10	0
300	15	66	10	0	10	0
400	25	70	10	0	10	0
500	15	80	10	0	8	0
600			10	0	10	0
700			8	0	10	0
800					10	0
900					10	0
1000					10	0
Tremors.....	Yes		No		No	
Dehydrochlorination rate..	12,515		75.6		83.0	

chlorophenyl)-methane cause tremors and they are definitely less toxic than DDT.

As illustrated in table 2 and figure 1-B, replacing the chlorine atom in para position of the phenyl ring by bromine and iodine, as in 2,2-bis-(p-bromophenyl)-

1,1,1-trichloroethane and 2,2-bis-(p-iodophenyl)-1,1,1-trichloroethane, does not effect the toxicity or the toxic manifestations of DDT, whereas both are

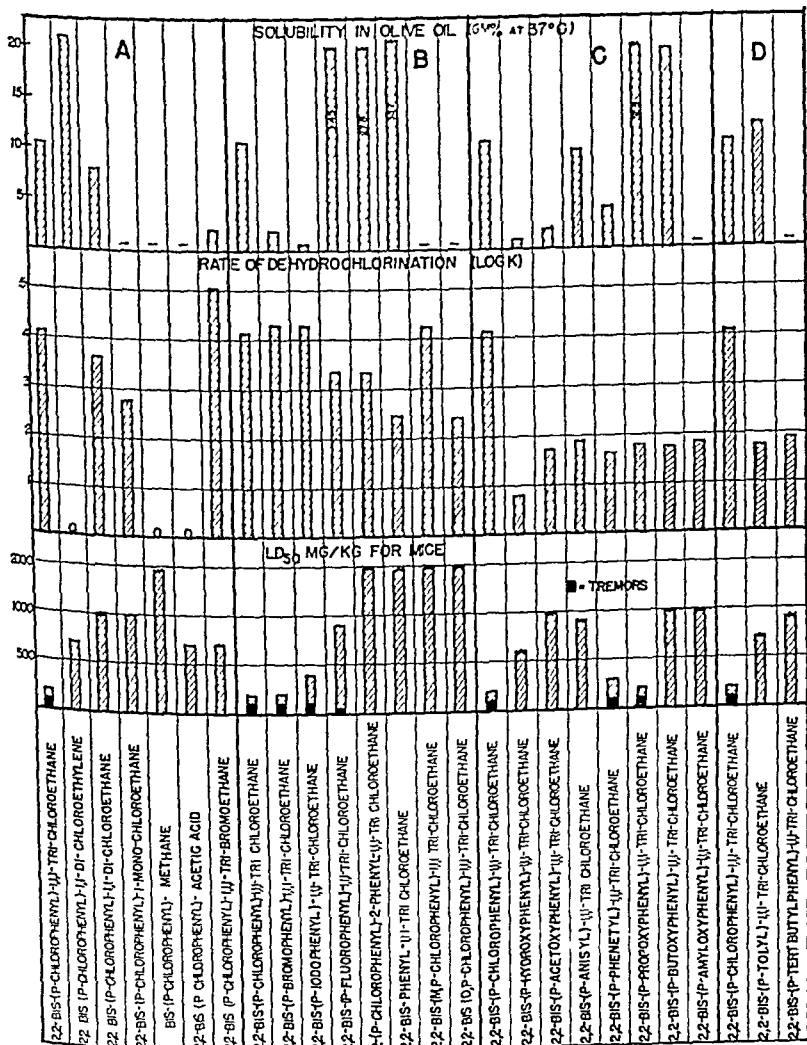


Fig. 1 gives the solubility of DDT and 22 DDT derivatives in olive oil (gm. % at 37°C.), their rate of dehydrochlorination (expressed in logarithms), their LD₅₀ for mice, and indicates those which produce tremors in mice.

attenuated by the introduction of fluorine, as in 2,2-bis-(p-fluorophenyl)-1,1,1-trichloroethane. The substitution of one or both chlorine atoms by hydrogen,

2-(p-chlorophenyl)-2-phenyl-1,1,1-trichloroethane and 2,2-bis-phenyl-1,1,1-trichloroethane, or the presence of hydrogen in para position with one chlorine atom in meta or ortho position, as in 2,2-bis-(m,p-chlorophenyl)-1,1,1-trichloroethane and 2,2-bis-(o,p-chlorophenyl)-1,1,1-trichloroethane, reduces the toxicity and neurological manifestations of DDT.

As shown in table 3 and figure 1-C, replacing chlorine by a hydroxylic group, as in 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, abolishes the characteristic action of DDT. This is not restored by esterification with acetic acid, as in 2,2-bis-(p-acetoxyphenyl)-1,1,1-trichloroethane, and the same holds true for the methyl, butyl and amyl ethers, 2,2-bis-(p-anisyl)-1,1,1-trichloroethane, 2,2-bis-(p-butoxyphenyl)-1,1,1-trichloroethane, and 2,2-bis-(p-amyltoxyphenyl)-1,1,1-trichloroethane. On the other hand, the ethyl and propyl ethers, 2,2-bis-(p-phenetyl)-1,1,1-trichloroethane and 2,2-bis-(p-propoxyphenyl)-1,1,1-trichloroethane, resemble DDT very closely in toxicity and neurological manifestations in mice.

As indicated in table 4 and figure 1-D, replacing chlorine by alkyl groups, as in 2,2-bis-(p-tolyl)-1,1,1-trichloroethane and 2,2-bis-(p-tertiary-butylphenyl)-1,1,1-trichloroethane, decreases the toxicity of DDT markedly and abolishes the neurological manifestations in mice.

Fleck and Haller (16), Martin and Wain (17) and Siegler and Gertler (18) suggested that the dehydrochlorination of DDT and similar compounds may be involved in the toxic action of DDT against insects, and Cristol (15) showed that the rate of dehydrochlorination of o,p'-DDT is $\frac{1}{87}$ that of p,p'-DDT which is the principal insecticide of technical DDT.

As illustrated in figure 1 and tables 1, 2, 3 and 4, this holds true to a certain extent also for the toxic manifestations of DDT derivatives in mice. For the compounds which produce similar neurological manifestations as DDT, the rate of dehydrochlorination is as follows:

2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane (DDT).....	12,515
2,2-bis-(p-bromophenyl)-1,1,1-trichloroethane.....	18,760
2,2-bis-(p-iodophenyl)-1,1,1-trichloroethane.....	19,800
2,2-bis-(p-fluorophenyl)-1,1,1-trichloroethane.....	2,319

The last of these compounds is also distinctly less effective than the others.

In contrast, 2,2-bis-(p-chlorophenyl)-1,1,1-tribromoethane has a rate of dehydrobromination of 127,000 although it is devoid of the toxic manifestations characteristic for DDT, but, as pointed out before, this may be due to its very limited stability.

On the other hand, 2,2-bis-(p-phenetyl)-1,1,1-trichloroethane and 2,2-bis-(p-propoxyphenyl)-1,1,1-trichloroethane, which resemble DDT very closely in their toxicity and neurological manifestations, have the low rates of dehydrochlorination of 43.3 and 62.0, respectively, whereas some ineffective compounds, namely 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane and 2-(p-chlorophenyl)-2-(phenyl)-1,1,1-trichloroethane, have such high values as 4035 and 2200, respectively. In the majority of cases, however, ineffective compounds have a rather low rate of dehydrochlorination, namely:

2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane.. . . .	563
2,2-bis-phenyl-1,1,1-trichloroethane	272
2,2-bis-(o,p-chlorophenyl)-1,1,1-trichloroethane	255
2,2-bis-(p-tertiary butylphenyl)-1,1,1-trichloroethane	83
2,2-bis-(p-anisyl)-1,1,1-trichloroethane	76.8
2,2-bis-(p-tolyl)-1,1,1-trichloroethane	75.6
2,2-bis-(p-amylxyphenyl)-1,1,1-trichloroethane	73.2
2,2-bis-(p-butoxyphenyl)-1,1,1-trichloroethane	59.1
2,2-bis-(p-acetoxyphenyl)-1,1,1-trichloroethane	50.8
2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane	7.0

It appears, therefore, that there is some indication that the rate of dehydrochlorination is affiliated with the toxic manifestations of DDT although it certainly is not a strict rule.

Since one of the factors influencing the pharmacodynamic action of chemicals is their lipid solubility, it was thought that the solubility of DDT and its derivatives in olive oil might give a lead in the interpretation of differences in their action. Luger and associates (19) assumed that the lipid solubility of DDT was affiliated with the trichloroethane group and that it was responsible for the affinity of DDT to the nervous system, similar to the relation of lipid solubility (partition coefficient oil/water) of chlorinated aliphatic hydrocarbons to their narcotic action, and a similar assumption was made by Martin and Wain (17). On the other hand, von Oettingen and associates (20) found that the brain and spinal cord of a goat repeatedly fed large doses of DDT dissolved in vegetable oil contained only small amounts of DDT, namely 15.5 and 17.4 mg. per cent, respectively, in contrast to the omentum fat which contained as much as 1121 mg. per cent. Similarly, Woodard and Ofner (21) found in rats fed various levels of DDT with the diet that it is stored to a large extent in the body fat from which it is mobilized upon starvation of the animals. It is, therefore, apparent that a great fat solubility may attenuate the toxicity of DDT and its derivatives by promoting their storage in the fat tissue and thus preventing their action on other structures.

As illustrated in tables 1, 2, 3 and 4 and figure 1, the solubility of DDT in olive oil is 10.5 per cent whereas the solubility of the ineffective compounds is as follows:

	Per cent
2,2-bis-(p-tolyl)-1,1,1-trichloroethane	12.1
2,2-bis-(p-chlorophenyl)-1,1,1-dichloroethane	21.1
2-(p-chlorophenyl)-2-phenyl-1,1,1-trichloroethane	27.8
2,2-bis-phenyl-1,1,1-trichloroethane	33.7
2,2-bis-(p-fluorophenyl)-1,1,1-trichloroethane	45

The last compound is only slightly less effective than DDT. On the other hand, 2,2-bis-(p-bromophenyl)-1,1,1-trichloroethane and 2,2-bis-(p-iodophenyl)-1,1,1-trichloroethane, which are as effective as DDT, are very little soluble in olive oil, namely 2.0 and 0.5 per cent, respectively. The ineffective 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, 2,2-bis-(p-acetoxyphenyl)-1,1,1-trichloroethane, and 2,2-bis-(p-chlorophenyl)-1,1,1-tribromoethane are also little soluble in olive oil, namely 0.9, 1.9 and 2.0 per cent, respectively. Of the five alkyloxyphenyl derivatives of DDT the oil solubility of 2,2-bis-(p-anisyl)-

1,1,1-trichloroethane and 2,2-bis-(p-phenetyl)-1,1,1-trichloroethane is relatively low, namely 9.7 and 3.9 per cent and that of 2,2-bis-(p-propoxyphenyl)-1,1,1-trichloroethane and 2,2-bis-(p-butoxyphenyl)-1,1,1-trichloroethane is high, namely 32.5 and 19.5 per cent, respectively. It should be pointed out that of these the methoxy and butoxy compounds are little toxic and cause no tremors, in contrast to the ethoxy and propoxy compounds which are about as effective as DDT in both respects. It is, therefore, apparent that the oil solubility alone is not a determining factor for the neurological manifestations of DDT, nor for its toxicity.

In view of the very low solubility of all these compounds in water their solubility in oil might be a determining factor for their absorption from the gastrointestinal tract. The comparatively low toxicity of 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane could be due to its low solubility in olive oil which is only 0.9% and hence to poor absorption from the intestinal tract. On the other hand 2,2-bis-(p-iodophenyl)-1,1,1-trichloroethane which is nearly as toxic as DDT, causing the same toxicological picture, is only soluble in olive oil to the extent of 0.5%. It is therefore not very likely that the lower toxicity of the p-hydroxyphenyl compound is solely due to delayed absorption because of its low solubility in olive oil.

Attempts to measure the *effect of these substances on the surface tension* failed because of their insolubility in water, and their effect on the surface tension of oil gave inconsistent or negative results.

Attempts to study their *reactivity with amino and imino groups* as with pyridine and piperidine, similar to the procedure used by Tronov (22) and Clarke (23) with chlorinated aliphatic hydrocarbons, failed because no combination between DDT and these compounds took place. The same holds true for *reactions between DDT and sulfhydrylic compounds* (thioglycolic acid and cysteine) and hydroxylic compounds like hydroquinone. It is, therefore, not very likely that the pharmacodynamic action of DDT is linked to its reaction with cellular constituents of this chemical character.

It appears reasonable to assume that introduction of certain radicals into the phenyl ring of DDT will not only affect the physico-chemical properties of the latter but will also alter its *fate in the metabolism* and the *rate of detoxication* of such derivatives, and, in some instances, may produce also a *physiological antagonism*.

It is understandable that a compound like 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, which is insoluble in water and little soluble in oil, is poorly absorbed and that because of the free hydroxylic group it may easily conjugate with sulfuric or glucuronic acid. For the same reason the acetoxy derivative, 2,2-bis-(p-acetoxyphenyl)-1,1,1-trichloroethane, which should be readily hydrolyzed with the formation of the p-hydroxyphenyl derivative, may be little toxic. Similarly, it appears not unlikely that the alkyl groups in para position of the phenyl ring, as in 2,2-bis-(p-tolyl)-1,1,1-trichloroethane and 2,2-bis-(p-tertiary-butylphenyl)-1,1,1-trichloroethane, undergo oxidation to carboxylic acid derivatives of DDT which may conjugate with glycine, although neither question has been investigated as yet. On the other hand, it is surprising that the alkyloxy derivatives of DDT differ so much in their toxicity and nervous

manifestations. As illustrated in table 3 and figure 1, 2,2-bis-(p-anisyl)-1,1,1-trichloroethane, 2,2-bis-(p-butoxyphenyl)-1,1,1-trichloroethane, and 2,2-bis-(p-amyloxyphenyl)-1,1,1-trichloroethane are little toxic and cause no tremors in mice, in contrast to 2,2-bis-(p-phenetyl)-1,1,1-trichloroethane and 2,2-bis-(p-propoxyphenyl)-1,1,1-trichloroethane which differ very little from DDT in their toxicologic manifestations. Of these, the methoxy compound may be unstable and readily converted to the ineffective p-hydroxyphenyl derivative, as indicated by the findings of Treupel (24) that the alkyl ethers of p-aminophenol are less effective methemoglobin formers than the mother substance, decreasing in their efficiency in this respect from the methyl to the amyl derivative. From this point of view of the stability it is difficult to understand why 2,2-bis-(p-butoxyphenyl)-1,1,1-trichloroethane and 2,2-bis-(p-amyloxyphenyl)-1,1,1-trichloroethane should be ineffective in contrast to 2,2-bis-(p-phenetyl)-1,1,1-trichloroethane and 2,2-bis-(p-propoxyphenyl)-1,1,1-trichloroethane. In order to appraise the influence of the different alkyl radicals on the toxicity of such derivatives of DDT, the toxicity of the methyl, ethyl, propyl, butyl and amyl ethers of phenol was studied in mice, using the same technic as described above. As illustrated in table 5, the toxicity of phenol is greatly decreased by closure of the hydroxylic group by alkyl radicals but the differences between the various ethers are better illustrated by their toxicological picture than by their L.D.₅₀. With all doses of phenol the animals showed excitement shortly after the administration and within a short time they developed tremors which passed into twitchings and, with the higher doses, into convulsions which with fatal doses ended rapidly in death. If the convulsions were not fatal the animals recovered and were apparently normal the next day. The L.D.₅₀ of phenol was determined as 300 mg./kg. The methoxy derivative (anisole) caused with the lower doses only moderate depression but with higher doses primary excitement, then progressive depression, rapid and labored respiration and finally coma and death, the animals showing marked cyanosis in the final stage of the poisoning. The L.D.₅₀ of anisole was determined as 2800 mg./kg. The ethoxy compound (phenetole) was slightly more toxic, the L.D.₅₀ being 2200 mg./kg.; shortly after the administration the animals became depressed and with larger doses they passed into deep narcosis in 2-3 hours, and the majority of the animals died in a comatose condition within 1-2 days. The L.D.₅₀ of propoxyphenyl was 3400 mg./kg.; shortly after the administration the animals became depressed and passed into narcosis and later into coma, but with nonfatal doses the animals showed only moderate drowsiness the next day. The L.D.₅₀ of butoxyphenyl was 3200 mg./kg.; shortly after the administration the animals became depressed and soon became narcotized, and with larger doses they passed into convulsions and were still in a state of stupor the following day. The L.D.₅₀ of amyloxyphenyl was 2200 mg./kg.; after a short period of hyperactivity the animals developed progressive depression, dyspnea, convulsions, paralysis of the hind legs, and coma which ended fatally or from which the animals recovered very slowly. These experiments show that the phenolic ethers have definite narcotic properties which are especially marked with the higher homologues, butoxyphenyl and amyloxyphenyl. It seems likely that the narcotic action of

these may antagonize the neurologic manifestations seen with the ethoxy and propoxy derivatives of DDT, whereas with the latter it is not sufficiently marked to suppress the tremors and twitchings.

It is apparent, therefore, that for the understanding of the relation between the chemical constitution of DDT and its derivatives and their pharmacodynamic action, their fate in the metabolism has to be investigated. Such studies are being carried out at present.

TABLE 5
Oral toxicity of phenol and alkyl phenols for mice

DOSE mg./kg.	PHENOL		METHOXY- PHENYL		ETHOXY- PHENYL		PROPOXY- PHENYL		BUTOXY- PHENYL		AMYLOXY- PHENYL	
	Death	Per cent	Death	Per cent	Death	Per cent	Death	Per cent	Death	Per cent	Death	Per cent
300	3/10 (2/10)	30 (60)	—	—	—	—	—	—	—	—	—	—
400	5/10 (3/10)	50 (80)	0/10	0	—	—	—	—	—	—	0/10	0
500	7/10 (1/10)	70 (80)	0/10	0	—	—	—	—	—	—	1/10	10
600	5/10 (1/10)	50 (60)	0/10	0	—	—	—	—	—	—	0/10	0
700	8/10 (2/10)	80 (100)	0/10	0	—	—	—	—	—	—	0/10	0
800	(figures in parentheses are late death, after 5-7 days)		0/10	0	—	—	—	—	—	—	0/10	0
900			0/10	0	—	—	—	—	—	—	—	—
1000			2/10	20	—	—	—	—	—	—	0/10	0
1100			1/10	10	—	—	—	—	—	—	1/10	10
1200			1/10	10	0/10	0	—	—	—	—	0/10	0
1300			2/8	25	—	—	—	—	—	—	2/10	20
1400			0/10	0	0/10	0	—	—	—	—	3/8	37
1500			0/10	0	—	—	—	—	—	—	2/10	20
1600			0/10	0	1/10	10	—	—	—	—	2/10	20
1700			1/10	10	—	—	—	—	—	—	3/10	30
1800			1/8	13	1/10	10	—	—	1/10	10	3/10	30
2000			2/10	20	8/10	80	1/10	10	0/10	0	4/10	40
2200			2/10	20	5/10	50	3/10	30	1/10	1	4/10	40
2300			—	—	—	—	—	—	—	—	5/10	50
2400			1/10	10	7/10	70	1/10	10	3/10	30	4/8	50
2600			3/10	30	7/10	70	3/10	30	1/10	10		
2800			4/8	50	8/8	100	1/10	10	3/10	30		
3000							3/10	30	4/10	40		
3200							2/10	20	6/10	60		
3400							4/10	40	6/10	60		
3600							6/8	75	6/8	75		

SUMMARY AND CONCLUSIONS

1. The toxicity and toxicological manifestations of 22 derivatives of DDT have been investigated.
2. It has been shown:
 - a. that the characteristic action of DDT depends upon the presence of the trichloroethane group,

- b. that it also depends upon substitution of the hydrogen atom in para position of the phenyl ring by halogen,
 - c. that the chlorine in this position can be replaced by bromine, iodine, fluorine, and the ethoxy and propoxy radicals without attenuating materially the toxic manifestations of DDT, and
 - d. that substitution of chlorine by a hydroxy, acetoxy, methoxy, butoxy or amyloxy group yields ineffective compounds.
3. The relation of these findings to the rate of dehydrochlorination and their solubility in oil is discussed.
4. It has been shown that introduction into the phenyl ring of a butoxy and amyloxy group instead of chlorine produces a physiological antagonism.
5. It is suggested that the inactivity of some of the DDT derivatives studied may be due to their fate in the metabolism.

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